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DEVELOPMENT OF ANTIBACTERIAL STRUCTURES AND FILMS USING GROUND PLANTS, EXTRACTS/ESSENTIAL OILS

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Ce mémoire intitulé:

DEVELOPMENT OF ANTIBACTERIAL STRUCTURES AND FILMS USING GROUND PLANTS, EXTRACTS/ESSENTIAL OILS

présenté par: ABDALI Hanan

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RÉSUMÉ

Les recherches portant sur les emballages alimentaires ont été principalement ciblées sur l'amélioration de la sécurité sanitaire et de la qualité nutritionnelle de l'aliment. Selon les normes internationales, un emballage approprié fournit également une plus longue durée de vie du produit alimentaire. De nouvelles stratégies d'emballages actifs sont en plein essor dans l'industrie de l'emballage, où de nouveaux matériaux multifonctionnels et l'utilisation d'agents antimicrobiens naturels gagnent un intérêt croissant. Le développement de nouveaux matériaux, en particulier les matériaux innovants antimicrobiens et naturels peut aider à répondre à ces besoins associés à d'autres fonctions de l'emballage telles que: la protection et la conservation des aliments, le marketing et la communication intelligente pour les consommateurs. Les plantes médicinales comme le clou de girofle, le thym, le romarin, la citronnelle, la cannelle et autres ont la capacité d'inhiber la croissance d'une vaste sélection de microorganismes pathogènes du fait de la présence d'huiles essentielles (HÉ) et de leurs composés. Les propriétés antimicrobiennes des HÉ et des extraits de plantes sont connues et utilisées depuis de nombreuses années. Néanmoins, certaines de ces HÉ et leurs composés ont montré des degrés variables de toxicité et d'effets indésirables sur la santé humaine. Les résultats de l'analyse thermogravimétrique (TGA) de notre étude ont clairement montré que lors de l'étude de la libération du thymol des films de LDPE et LLDPE vers la surface au cours du temps et à des températures élevées, le taux d'évaporation du thymol augmente pendant le mélange en extrusion bi vis et en mélangeur interne. Par conséquent, il a été décidé de travailler avec les poudres des plantes elles-mêmes, à la place de leurs huiles essentielles. Dans la première étape, les activités antimicrobiennes contre la bactérie *Escherichia coli* (*E. coli* DH5α) des poudres de plantes moulues dont la sauge, le clou de girofle, les feuilles de clou de girofle, la citronnelle, les graines de moutarde noire, les feuilles de menthe sauvage et les feuilles de thym ont été évalués et comparées. La poudre de clou de girofle (*Syzygium aromaticum*) a montré l'activité antimicrobienne la plus élevée par rapport aux autres plantes utilisées dans cette étude. La concentration minimale bactéricide (MBC) de la poudre de clou de girofle contre *E. coli* a également été mesurée en utilisant deux méthodes différentes de stérilisation. Les résultats ont montré que les valeurs de CMB de la poudre de clou

de girofle avant stérilisation étaient de 200 mg/ml. Après stérilisation avec deux méthodes différentes, les valeurs étaient ≥ 50 mg/ml.

La deuxième étape de cette étude était d'étudier l'effet inhibiteur du clou de girofle en poudre contre la croissance d'une bactérie à Gram négatif : *Escherichia coli* (*E. coli* DH5α) et deux bactéries à Gram positif : *Listeria innocua* (*L. innocua* SPQ3284) et *Staphylococcus aureus* (*S. aureus* 54-73). La concentration minimale inhibitrice (CMI) et la concentration minimale bactéricide (CMB) ont été déterminées en utilisant la technique de dénombrement sur gélose. Les résultats ont montré que la poudre de clou de girofle a eu un effet inhibiteur de croissance contre les bactéries testées. Les valeurs des CMI et CMB ont clairement montré que la poudre de clou de girofle possède une activité antimicrobienne plus élevée contre S. aureus comparativement à E. coli et L. innocua. Les valeurs des CMI et CMB de la poudre de clou de girofle contre S. aureus sont de 100 mg/ml et 120 mg/ml, respectivement. La distribution de taille des particules de poudre de clou de girofle a été mesurée et la taille des particules a été réduite en utilisant la technique de broyage à sec.

Dans la dernière étape, l'activité antimicrobienne de films et de fibres contenant de la poudre de clou de girofle a été évaluée. Les nanofibres de Poly (caprolactone ε-) (PCL) et clou de girofle en poudre ont été préparées par le processus d'électrofilage, tandis que pour les films, la poudre de clou de girofle a été intégrée par imprégnation dans le polyéthylène à basse densité (LDPE). L'activité antimicrobienne de la poudre de clou de girofle n'a pas montré un effet antimicrobien élevé lorsque incorporée dans les fibres électrofilées de PCL. Ceci a été attribué à une mauvaise solubilisation des poudres dans les solvants (DCM: DMF). Le film de LDPE imprégné deux fois avec la poudre de clou de girofle a montré les effets antimicrobiens les plus élevés contre la bactérie *E. coli*.

ABSTRACT

Food packaging research has been mainly targeted towards improving food quality and safety. Based on international standards proper food packaging provides longer product shelf life. New active packaging strategies represent the focal point in development in the packaging industry, where new multifunctional materials and the use of natural antimicrobial agents are gaining increasing interest. The development of new materials, and particularly innovative natural antimicrobial materials, may assist to address these requirements coupled with other packaging functions such as: food protection and preservation, marketing and smart communication to consumers. Medicinal plants like clove, thyme, rosemary, lemon grass, cinnamon and others have the ability to inhibit the growth of a vast selection of pathogenic microorganisms due to the presence of essential oils (EOs) and their compounds. The antimicrobial properties of EOs and plant extracts have been known and used for many years. Nevertheless, some of these EOs and their compounds have shown varying degrees of toxicity and adverse effects on human health. The thermogravimetric analysis (TGA) results in our study have clearly demonstrated that while studying the release of thymol from LDPE and LLDPE films to the surface of the films over time, at high temperatures, the evaporation rate of thymol increased during blending with LDPE and LLDPE by using twin-screw extruder and Brabender. Therefore, it was decided to work with the plant powders themselves instead of the essentials oils. In the first step, the antimicrobial activities of ground powdered plants such as sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf against *Escherichia coli* (*E. coli (*DH5α)) were evaluated and compared. The clove bud powder (*Syzygium aromaticum*) showed the highest antimicrobial activity compared to the other ground plants used in this study. Then the minimum bactericidal concentration (MBC) of the clove bud powder against *E. coli* was measured by using two different methods of sterilization. The results showed that, the MBC values of the clove bud powder without sterilization was 200 mg/ml and with two different methods of sterilization were ≥ 50 mg/ml.

The second step of this study was to investigate the growth inhibitory effect of the powdered clove bud against one Gram-negative bacteria *Escherichia coli (E. coli)* (DH5α) and

two Gram-positive *Listeria innocua (L. innocua)* (LSPQ3284) and *Staphylococcus aureus (S. aureus)* (54-73) microorganisms, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) tests were carried out using the agar plate counting technique. The findings indicated that the clove bud powder had a growth inhibitory effect against the tested bacteria. The MIC and MBC values have clearly shown that the clove bud powder has higher antimicrobial activity against *S. aureus* than *E. coli and L. innocua*, where the MIC and MBC values of clove bud powder against *S. aureus* were 100 mg/ml and 120 mg/ml, respectively. The particle size distribution of clove bud powder was measured and the size of the particle was reduced by using the dry milling technique.

In the final step, the antimicrobial activity of the clove bud powder was evaluated when applied into films and fibers. Poly (ε- caprolactone) (PCL) and clove bud powder was produced via the electrospinning process, then, the clove bud powder was embedded into the low-density polyethylene (LDPE) film in this study. The antimicrobial activity of the clove bud powder did not show a strong antimicrobial effect when incorporated into PCL electrospun fibers, which was attributed to the improper dissolution of powders in the solvents (DCM: DMF). The LDPE film embedded with the clove bud powder when coated twice, showed the strongest antimicrobial effects against *E. coli* bacteria.

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CHAPTER 1 INTRODUCTION

When food is exposed during slaughter, processing, packaging, shipping and storage, it can lead to contamination, where spoilage and growth of pathogenic microorganisms can occur during favorable environments [1,2]. There are several research methods underway to find the best possible solutions in limiting the problems related to foodborne microorganisms and food spoilage. For instance, antimicrobial (AM) packaging is a modern development technique that incorporates the antimicrobial agent into a polymer film to inhibit the activities of targeted microorganisms [2,3,5]. Current methods of AM packaging provide good protection and long shelf life, and also prevent food from various risks including microbial spoilage [6,7,8]. As a result, there are many published reviews describing the highlighting antimicrobial properties, mode of action, possible packaging materials, spectrum of activity, current uses and new applications of AM agents [5,6,7,8,9,10,11,12,13,14,15,16,17]. However, the great majority of these AM agents are chemically based, and the main concern in using chemical AM agents are their possible side effects. Many studies have shown the impact on human health in the continuous consumption of food applied with such chemical AM agents [18,19].

In recent years, there has been great interest in using natural additives of plant origin in food packaging technologies such as AM agents, since it is widely regarded that phytogenic substances are healthier, deemed less chemically hazardous, and generally regarded as safe. Using plant extracts and/or essential oils (EOs) in packaging materials as natural AM agents, provides food with adequate protection from the growth of pathogenic organisms. Under appropriate conditions, the use of the plant extracts and essential oils in packaging materials helps to control microbial growth, increase food safety, and extend product shelf life [18].

The antimicrobial properties of plant extracts and essentials oils in plants have been recognized for many years, where several studies have shown the effectiveness of plant extracts and essential oils as AM agents [18,19,20]. For example, it was found that, EOs, ethanol and aqueous extracts from clove and cinnamon were able to inhibit the growth of foodborne pathogenic bacteria such as *Staphylococcus aureus, Vibrio parahaemolyticus,* and *Listeria monocytogenes* [18]. Up to now, many studies have investigated the antimicrobial activity of

EOs and their compounds as antimicrobial agents but not the herbal plants themselves, like the properties of the clove bud.

In this regard, the main objective of this project is to develop an effective strategy for incorporating the clove bud as an antibacterial agent in food packaging materials. The first step in achieving this goal was to evaluate the antimicrobial activity of a selection plant species for example; sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf and determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the clove bud for three different types of bacteria. Additionally, the effectiveness of the antimicrobial activity of the clove bud, the particle size distribution were measured and size of the particle was reduced by using the dry milling technique in the second step. In the final step of this work, the clove bud was applied into films and fibers where its antimicrobial activity evaluated. To this purpose, PCL and clove bud powder produced via the electrospinning process and clove bud powder embedded into the LDPE film in this study, were evaluated for their antibacterial properties.

This thesis comprises of eight chapters. In the first chapter, active packaging systems, especially antimicrobial food packaging systems, are briefly introduced. An overview of natural antimicrobial packaging, phytogenic additives such as plant extracts, EOs, the chemical composition and mode of action are detailed in the second chapter, while materials and methods used in this study are described in the third chapter. The fourth chapter, briefly details the order and organization of my work reported in this thesis. The fifth chapter, discusses the inhibitory effects of seven kinds of powdered plant species against *Escherichia coli* (*E. coli*) (DH5 α), the MBC of the clove bud powder and then its AM activity monitored by the electrospun PCL and clove bud powder blends dissolved in a mixture of (DCM: DMF) (50:50) v/v are determined. In chapter six, the antimicrobial activity of the clove bud powder against foodborne bacteria, its MIC, MBC and its efficiency when coated onto or incorporated into the polymer films or sheets were studied and compared. Chapter seven, discusses the migration kinetics of thymol from LDPE and LLDPE to the surface of the film over time by using the FTIR–ATR spectroscopy test, characterization of the AM activity of the electrospun PCL and thymol fibers and then a thorough discussion regarding the most important factors affecting the AM activity of clove bud powder,

in chapter eighth. Finally, in the ninth chapter the conclusions and recommendations are given.

1.1 Background

Role of Packaging for Food

Packaging protects food against all adverse external influences for example, environmental conditions, physical damage, biological contamination, that can alter the properties of the product; for convenience, and to communicate information on the package to a consumer [21,22]. Therefore, the main functions of packaging are containment, protection, convenience, and communication [22,23]. All products must be contained before they can be moved to a new location, without containment, product loss and pollution would be widespread [22,23,24].

The fundamental functions of the package is to protect its contents from external environmental effects like gases, dust, temperature variation, water, moisture, light, odors, shocks, and micro-organisms, as well as to protect the food from physical damage such as vibrations arising from transportation modes arising from various transportation modes and storage in warehouses and storage in warehouses [24,25].

Many aspects of convenience are important in package design such as ease of access, handling, reseal-ability, disposability, product visibility, and microwave-ability [25,26]. It is important that the messages are clearly received by consumers [25,26,27]. Therefore, the package must contain ingredient and nutrition information required by law such as nutrition labeling, standardized serving sizes, and nutrient reference values which consumers require to understand the relationship between nutrition, diet, health and other information [25,26,28].

Packaging materials are comprised of a combination of several materials with unique properties that can ensure both the safety and quality of the food content during the manufacturing, processing, handling and storing, whereby, it is finally sold to the consumer [21,25,27]. As a result, the design and construction of the packing material plays a primary role

in keeping a product safe and fresh from external influences and damage during distribution [21,22].

New Food Packaging Technologies

The demand for handling food and its safe consumption has intensified in recent years. The frequent incidences of food poisoning from contaminated food worldwide have heightened research and development in the field of food safety to improve human food storage and consumption [21,28]. These widespread contaminations have been attributed to a variety of factors, such as the increase in global trade through the export and import of food products, changes in current methods of food production, changing modern lifestyles, changes in food consumption, and the emergence of new pathogens [28,29]. This has resulted in major challenges in food safety and quality [28,29,30]. Recently, there are many innovative ways to prevent or inhibit microbial growth in food while maintaining quality, freshness, and safety [28,31]. Therefore, a variety of advanced new packaging technologies such as active, intelligent or smart packaging have been developed to protect food products from environmental conditions, make distribution easier, extend the shelf life and improve the safety of food while providing information and convenience to the consumer [23,28,30].

Active Food Packaging

Active packaging (AP) has been defined as a system in which the product, the package, and the environment interact in a positive way in order to extend the shelf life of food and achieve certain desired outcomes [32,33]. The main objective of the active packaging is to change the condition of packaged food, to extend shelf life and improve the safety or sensory properties, while still maintaining the quality of the food [6,8,34].

The AP system for preservation and improving quality and safety of food can be classified into three categories: active scavenging systems (absorbers), active releasing systems (emitters) and controlled release packaging systems (AM agents) [6,12]. The internal atmosphere of the package can be controlled by certain active substances that can absorb (scavenge) or release

(emit) gases or vapors [12,34]. Some examples of AP systems include moisture scavengers, carbon dioxide scavengers, oxygen scavengers, ethylene scavengers, humidity absorbers or controllers, aroma emitters or absorbers and AM systems [8,12,35]. AP materials that possess the ability to release active compounds which enhance the quality and safety of a wide range of foods during extended storage are particularly important [6,36]. The release of active compounds by the packaging material plays an important role in determining the inhibitory effect on the spectrum of microorganisms [10,35,36].

Antimicrobial Food Packaging

Food Safety and AM Packaging

Deterioration of food products is caused both biologically and chemically. The major cause of biological deterioration in food is primarily due to the growth of microorganisms that spoil the food and render it unsafe or unsuitable for human consumption [11,37,38]. Microbial contamination due to yeasts, molds and bacteria results in degraded and deteriorated foodstuff which also reduces their quality and shelf life as well as presenting serious health issues upon consumption [11,37]. Therefore, the main concept in using antimicrobial substances in polymeric matrices is to kill or inhibit the growth of microorganisms thus extending the shelf life and safety of perishable packaged products [10]. AM packaging acts on the surface of foods where most of the spoilage and contamination occurs but also act on microorganisms that may be present in or on the packaging material itself [27,12,34].

Fish, poultry, meat, fruits and vegetables, dairy and bakery products are some examples of food that are prone to surface microbial spoiling and have been the focus for AM packaging systems [39]. There are various types of antimicrobial substances that have been used in synthetic and natural polymers such as enzymes, bacteriocins, organic acids and their salts, miscellaneous compounds like silver, zinc oxide, plant extracts and EOs like thymol, carvacrol, eugenol, [10, 38] (see Table 1.1).

Table 1.1 Antimicrobial agents and packaging materials used in food packaging [Adapted 10,38]

Antimicrobial Agent	Packaging Material	Application area
Potassium sorbate	LDPE	Cheese
	LDPE	LDPE
	MC/palmitic acid	Culture media
	MC/HPMC/fatty acid	Culture media
	MC/chitosan	Culture media
	Starch/glycerol	Chicken breast
Calcium sorbate	CMC/paper	Bread
Propionic acid	Chitosan	Water
Acetic acid	Chitosan	Water
Benzoic acid	PE-co-MA	Culture media
Sodium benzoate	MC/chitosan	Culture media
Sorbic acid anhydride	PE	Culture media
Benzoic acid anhydride	PE	Fish fillet
Imazalil		
Nisin (peptide)		
Lysozyme	LDPE	Bell pepper
	LDPE	Cheese
Glucose oxidase	Silicon coating	Culture media
	SPI, corn zein films	Culture media
Alcohol/thiol	PVOH, nylon, Cellulose	Culture media
	acetate, corn zein films	Culture media
Ethanol	Alginate	Fish
Reduced iron complex	Slica gel sachet	Culture media
	Silicon oxidase sachet	Bakery
BHT		
Chelating agents	Sachet	Bread
EDTA	HDPE	Breakfast cereal
Cinnamic	Edible films	Culture media
Caffeic		
p-coumaic acids	Starch/glycerol	
Grapefruit seed extracts	Nylon/PE, cellulose	Culture media
Hinokitiol		
Bamboo powder		
Rheum palmatum		
Coptis chinesis extracts		
CO_2	LDPE, cellulose	Culture media
SO_2		
	Calcium hydroxide sachet	Coffee
	Sodium metabisulfite	Grape

Types of Antimicrobial Food Packaging

The types of antimicrobial food packaging can be listed as follows [7] (see Figure 1.1).

1.2.2.1 Addition of Sachets/Pads Containing Volatile Antimicrobial Agents into Packages

Prototypes in AM packaging were in the form of sachets or pads containing the active ingredient that were enclosed or attached to the interior of the package [7]. Ethanol vapor generators, oxygen absorbers, and moisture absorbers are the main types of sachets used commercially [7]. Oxygen and moisture absorbers are used primarily in bakery products, pasta and meat packaging to inhibit oxidation and water condensation [24]. Even though, oxygen and moisture absorbers are not AM agents, they indirectly inhibit microbial growth [7]. For example, reducing the headspace oxygen in the package restricts the growth of aerobes or using moisture absorbers that reduce water activity and indirectly affect microbial growth on the food [7].

1.2.2.2 Direct Incorporation of Antimicrobial Agents into Packaging Materials

There are two ways to incorporate antimicrobial agents into packaging materials, one is the addition of antimicrobial agents into the melt polymer and the other is the addition into the wet polymer solution. The diffusion of antimicrobials from packaging materials has been widely reported due to their potential to provide higher quality and safety of food and many research efforts are focused on their development and implementation [4,7,37,40,41]. A study by Lee et al. (1998) the incorporated of 1% w/w grapefruit seed extract in LDPE films of 30 μm thickness by using blown film extrusion processing at 150 °C where it was discovered that grapefruit seed extract inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* [46]. Furthermore, the films successfully inhibited the growth rate of lactic acid bacteria when used for packing curled lettuce and soybean sprouts. Moreover, the growth rate of yeast and aerobic bacteria were considerably reduced [46]. For volatile antimicrobial agents like allylisothiocyanate, sulfur

dioxide, and EOs; packaging materials do not need to be in contact with the surface of the food [7,15]. On the other hand, for the diffusion of the non-volatile antimicrobial agents, packaging materials must be in contact with the surface of the food [15,42,43,44,45].

1.2.2.3 Coating or Absorbing Antimicrobials to Polymer Surfaces

This technique is used as an alternative to the incorporation of antimicrobial compounds such as enzymes that are sensitive to high temperature, during extrusion and cannot be used in polymer processing [40]. Accordingly, they are often coated onto the material after forming or are added to cast films [40,47]. For example, nisin / methylcellulose coatings applied on polyethylene films [7,47]. Proteins have an increased capacity for absorption due to their amphiphilic structure [40].

1.2.2.4 Immobilization of Antimicrobials by Ionic or Covalent Linkages to Polymers

Chemical methods of immobilizing the antimicrobial agents to polymers by ionic or covalent bonding only occurs if the antimicrobial agent and the polymer have functional groups [7]. Some such polymers include, nylon, polystyrene (PS), ethylene vinyl acetate (EVA), ethylene methyl acrylate (EMA), and ionomer. Organic acids, enzymes, polyamines, and peptides are also antimicrobial agents with functional groups [7,37].

1.2.2.5 Polymers that Are Inherently Antimicrobial

Chitosan and poly-L-lysine are cationic polymers, which are inherently antimicrobial and have been used in films and coatings. As AM agents, they interact with negative charges on the cell membrane and cause the leakage of their intracellular components [7]. Chitosan has recently been approved as a food ingredient by the FDA and is gaining popularity as a natural antimicrobial agent [6].

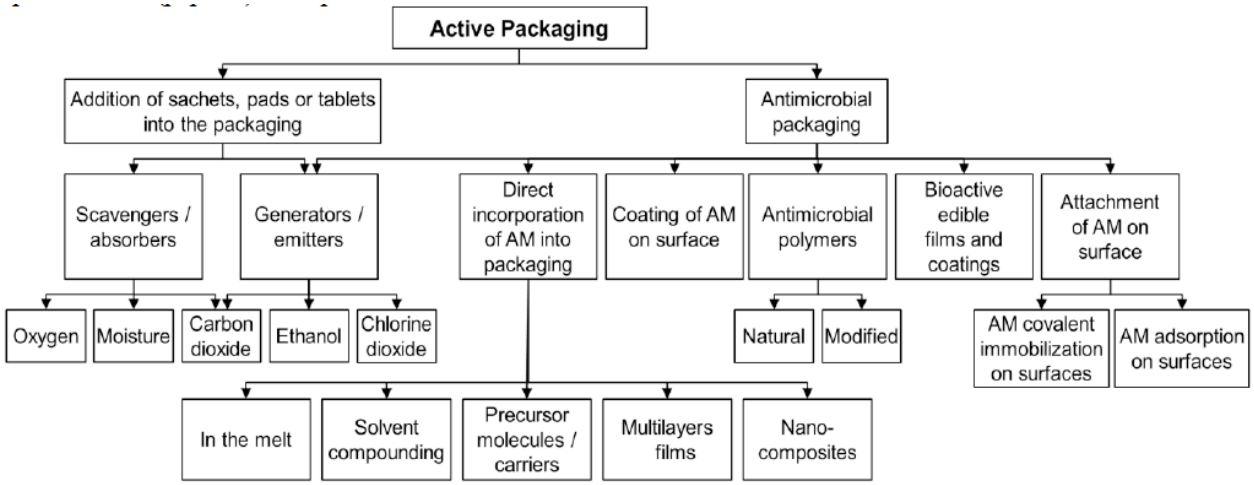

Figure 1.1 Classification of different active packaging systems [Adapted from 47]

Antimicrobial Packaging Systems

Antimicrobial food packaging systems represent either package / food systems or package / headspace / food systems. Some of the differences between the two systems are discussed below.

1.2.3.1 Package / Food Systems

In these systems, packaging materials, which are generally polymers, are in contact with a solid food product, or a low viscosity/liquid food without headspace [10]. Wrapped cheese, deli products and ready-to-eat meat products are examples of food packaged in this system [11,37]. The diffusion between the packaging material, food and partitioning at the interface are the main migration phenomena involved in this system (a non-headspace system) [11]. As shown in Fig. 1.2, an AM agent incorporated into the packaging material initially can migrate into the food through diffusion and partitioning [11].

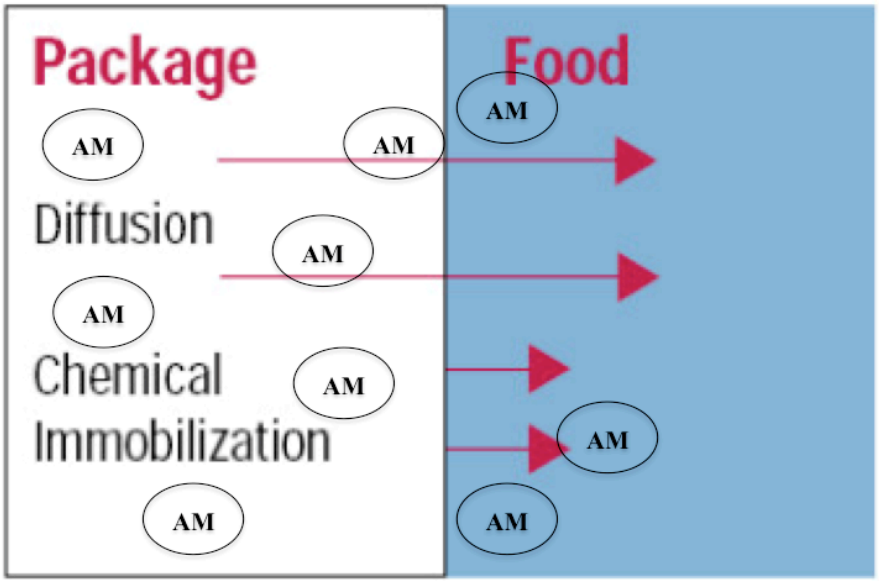

Figure 1.2 Package / food systems [Adapted from 10]

1.2.3.2 Package / Headspace / Food Systems

Flexible packages, bottles, cans, cups and cartons are examples of package / headspace / food systems [11]. Evaporation or equilibrated distribution of an AM agent among the head space, packaging materials and food are factors to be considered during the main migration in order to estimate the interfacial distribution of the AM agent [11]. In these systems, AM agents must be volatile so they can migrate through the headspace and air gap between the package and the food (see Figure 1.3), compared to a non-volatile substance that can only migrate through the contact area between the package and the food in the non-headspace system [11].

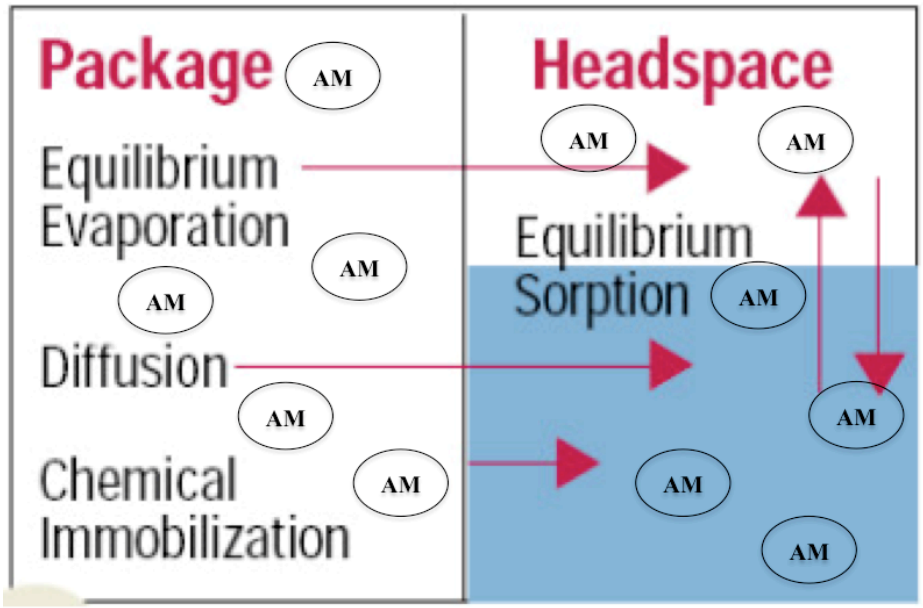

Figure 1.3 Package / headspace / food systems [Adapted from 10]

Design Factors for Antimicrobial Packaging

There are many factors that need to be considered when designing AM packaging systems. AM agents have a specific inhibition activity against particular pathogens; therefore the selection of an AM agent depends mainly on its activity against the target pathogens [6]. Important characteristic properties of an AM agent are water solubility, specific gravity, organoleptic properties, toxicity and resistance to microorganisms [11]. Other factors that should also be considered when designing AM packaging systems are the method of incorporation into the packaging, permeation and evaporation, controlled release, and the physio-mechanical properties of the packaging materials [48]. In addition, other characteristics of food products such as the composition and chemical nature, i.e. introduction pH, water activity including manufacturing, storage and distribution where conditions such as temperature are also important [49]. Finally, all these factors must be considered in accordance with the relevant regulations in order to design the most effective antimicrobial package.

The design of an AM packaging system also requires understanding and knowledge of controlled release technology and microbial growth kinetics [50]. In recent years, the migration of packaging components into food consumed, has received considerable attention that has generated improvements of the preservative and antimicrobial properties of AM agents [43,44,45]. Nevertheless, studies on the development of food packaging films with controlled release mechanisms are extremely limited. One of the more efficient methods of achieving appropriate controlled release to the food surface, are by incorporating AM agents into multilayers of the films (control layer/matrix layer/barrier layer). The innermost layer controls the rate of diffusion of the active agent, while the matrix layer contains the active agent and the barrier layer limits the migration of the agent towards the outside of the package [35,52, 51,53]. This concept of a controlled release in food packaging systems was applied first by Han and Floros (1998) followed by Lopez-Rubio et al (2004) [35,53]. Based on the work of Han and Floros (1998), Buonocore et al. (2003) developed two multilayer films that consisted of two external control layers and an inner layer with the AM agent [40,53].

Antimicrobial Agents

Definition of Antimicrobial Agents

Antimicrobial packaging is a promising form of active packaging that requires the presence of antimicrobials in order to kill microorganisms or inhibit their growth thereby extending shelf life and improving safety standards. There are various microorganisms such as bacteria, yeasts, and molds, etc., that degrade and deteriorate food. The growth of most of these organisms can be destroyed or inhibited by using different antimicrobial agents. The AM agents can be used either alone or in combination with each other within a polymeric matrix to attain more efficient antimicrobial properties. Different types of AM agents are either chemical or natural compounds that can be used in antimicrobial films, utensils, and containers [10,37,38] (see Table 1.2).

Table 1.2 Examples of the different types of antimicrobial agents for potential use in food packaging materials [Adapted from 10,37,47]

Antimicrobial Agents classes	Examples
Organic acide	Acetic acid, benzoic acid, p-aminobenzoic acid, citric acid, lactic acid, malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid
Organic acid salts	Potassium sorbate, sodium benzoate, potassium lactate
Organic acid anhydrides	Benzoic anhydride, sorbic anhydride
Inorganic acids	Phosphoric acid
Inorganic gases	Sulfur dioxide, chlorine dioxide
Alcohols	Ethanol
Amines	Hexamethylenetetramine (HMT)
Ammonium compounds	Silicon quaternary ammonium salt
Antibiotics	Natamycin
Antimicrobial peptides	Defensin, magainin, attacin, cecropin
Antioxidants	Butylatedhydroxyanisole (BHA)
Bacteriocins	Bavaricin, brevicin, carnocin, lacticin, mesenterocin, nisin, pediocin, sakacin, subtilin
Chelators	Citrate, conalbumin, ethylenediaminetetraacetate (EDTA), lactoferrin, polyphosphate
Enzymes	Chitinase, ethanol oxidase, β-glucanase, glucose oxidase, lysozyme, myeloperoxidase
Fatty acids	Lauric acid, palmitoleic acid, glycerol mono-laurate
Fatty acids ester	Monolaurin (lauricidin®)
Fungicides	Benomyl, imazalil
Metals	Copper, silver, zirconium, titanium oxide
Plants and spices	Allyl isothiocyanate (AITC), grapefruit seed extract, bamboo powder, rheum palmatum, coptischinensis extracts, cinnamic acid, caffeic acid, p-coumaic acid
Essential oils and plant-volatile components	Carvacrol, cineole, cinnamaldehyde, citral, p-cymene, estragole (methyl chavicol), geraniol, Hinokitiol (β-thujaplicin), linalool, terpineol, thymol, oregano, lemongrass
Natural phenols	Catechin, p-cresol, hydroquinones
Phenolic compounds	Butylatedhydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ)
Parabens	Ethyl paraben, methyl paraben, propyl paraben
Polysaccharides	Chitosan, konjac glucomannan
Oligosaccharides	Chitooligosaccharide
Miscellaneous	Reuterin, triclosan, nitrites, sulphites, and probiotics

Types of Antimicrobial Agents

1.3.2.1 Chemical Antimicrobial Agents

A chemical that possesses the ability to destroy or inhibit deterioration can be considered as a viable chemical antimicrobial agent [54]. Some chemical AM agents that have been widely used in recent years are sorbic acid, potassium sorbate, zinc oxide, sulfur dioxide chlorine dioxide and benzoic acid which are all used in the food industry [54]. However, using chemical AM agents in food packaging has certain drawbacks; they are usually distributed in the bulk of food at relatively large quantities often imparting off flavors and odors, and there is rising concern about the side effects of synthetic additives amongst consumers [7]. AM packaging systems that are primarily based on natural AM additives like essential oils (EOs) or/and plant extracts, have started to evolve with regard to these concerns [7,40].

1.3.2.2 Natural Antimicrobial Agents

In recent decades, the consumption of food containing natural preservatives is becoming popular. As a result, the usage of natural AM agents in foods and food packaging materials has gained credibility in the food industry. The most popular natural AM agents are essential oils/ plant extracts such as eugenol, carvacrol, thymol, oregano, lemongrass, linalool. Recent studies have investigated the use of plant extracts and/or essential oils in active packaging [18]. Using plant extracts/essential oils in the packaging materials under appropriate conditions prevents or limits many problems related to the growth of bacteria such as *Escherichia coli (E. coli)*, *Listeria monocytogenes (L. monocytogenes)*, *Staphylococcus aureus (S. aureus)*, *Salmonella typhimurium (S. typhimurium)* and *Bacillus cereus (B. cereus)* [18,19]. However, there are marked differences in the properties of AM agents due to both quantitative and qualitative variations of the plant extracts and/or EOs used. Hence, it is equally important that the correct identification of the botanical source and standardization of extraction methods are taken into account [55,56] (see Table 1.3).

Table 1.3 Plants species and main constituents of the respective essential oils [Modified from 55]

Plant origin	Plant species	Main compounds (%)
Thyme	*Thymus vulgaris*	Thymol (31.4%), p-cymene (17%), carvacrol (12.4%), γ-terpinene (11.1%).
Clove	*Syzygium aromaticum*	Eugenol (64%), eugenyl acetate (16.3%), caryophyllene (14.5%).
Cinnamon	*Cinnamomum verum*	Cinnamaldehyde (75.3%), coumarin (10.6%), cinnamic alcohol (3%).
Oregano	*Origanum compactum*	Carvacrol (36.5%), thymol (29.7%), p-cymene (24.3%), γ-terpinene (1.1%).
Rosemary	*Rosmarinus officinalis*	Carnosic acid, carnosol, rosmadial, genkwanin, rosmarinic acid
Lemongrass	*Cymbopogon citratus*	Geranial (45.7%), myrcene (3.9%), 6-methylhept-5-en-2-one (2.7%)

CHAPTER 2 LITERATURE REVIEW

Thyme and Clove Bud as a Natural Source of AM Agents

Herbal plant species and spices are known to contain a wide range of organic compounds capable of exhibiting AM activity [18,56]. These compounds are secondary metabolites, which they often have antimicrobial properties. The antibacterial properties of secondary metabolites were first evaluated in the late nineteenth century by De la Croix (1881), who employed essential oil vapors [58]. Natural extracts are obtained from different parts of plants such as flowers, buds, seeds, leaves, twigs, bark, wood, fruits, roots and comprise of variable mixtures, primarily terpenoids, phenolic compounds and a variety of esters, aldehydes, ketones, acids and alcohols [18,59]. Active components such as terpenoids and phenolic compounds are found in large amounts in plant extracts and EOs [57,58]. These EOs are also called volatile oils; but the chemical composition of these plants or herbs and concentration of active components varies greatly depending on their source [57,58]. Interest in ground plants and their extracts and/or EOs as well as their applications in food industry has been heightened in recent decades owing to negative consumer perception of synthetic agents [18,56,58]. Ground plants, their extracts and/or EOs have been used in cosmetology, perfumery, and medicine besides being added to foods as part of herbs or spices; furthermore, there are several studies in past decades focusing on their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties, which is why they are widely used in medicine and the food packaging industry [18,59,60]. Using plant extracts and/or the EOs in food packaging materials as natural AM agents in food preservation requires detailed knowledge about their properties like, the range of target microorganisms, the mode of action, the minimum inhibitory concentration (MIC), and the effect of food matrix components on the antimicrobial properties of the AM agents [61].

Thyme is a popular culinary herb belonging to the *Lamiaceae (Labiatae)* family; the genus *Thymus* is noted for its many species and varieties [62,63]. There are several species of *Thymus* that are identified as a source of thyme [64]. The most important species and widely

used as a flavoring is the *Thymus vulgaris*. Thyme contains EOs that are phenolic monoterpenoids for example; thymol ($C_{10}H_{14}O$ 2-isopropyl-5-methylphenol), carvacrol, p-cymene and γ-terpene [65]. Thymol is the major phenolic component in thyme where the high concentration of thymol is responsible for the strong antimicrobial activity and spicy smell [65]. Historically, the culinary and medicinal qualities of thyme have been used for centuries, where it was attributed with potential health benefits, antioxidant activity and AM effects that provides food with flavor and preservative qualities [65].

Clove is also known as *Syzygium aromaticum* and belongs to the myrtle family, *Myrtaceae*. The essential oil extracted from clove has many therapeutic effects, including kidney fortification, anti-vomiting, antispasmodic and anti-phlogistic. It is widely used in the pharmaceutical industry, especially in medicinal preparations for gum and teeth [66,67]. It is also used in the fragrance and flavoring industries [68,69]. The essential oil extracted from the clove bud has biological properties such as antibacterial, antifungal, insecticidal and antioxidant [68,69]. It is also used traditionally as flavoring agent and antimicrobial additive in food [69,70]. Some studies have reported that the leaf and bud oils were complex mixtures of numerous compounds with different antimicrobial properties; many of which are present in trace amounts [67, 68,70]. Clove contains EOs like phenylpropene phenol and the two major constituents in clove oil are eugenol ($C_{10}H_{12}O_2$ 2-Methoxy-4-(2-propenyl) phenol), and β-caryophyllene, where the high concentration of eugenol is responsible for the strong antimicrobial activity [66, 67, 69].

Thyme, clove, oregano, marjoram and their EOs are Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA) under the conditions of intended use [71]. Thymol is also approved as a food additive, particularly as a flavoring agent, in Europe (EC 2005) and USA (FDA 2005); it is also included in the European Union (EU) list of flavoring substances [71,72]. Clove and its EOs are also approved for use as over-the-counter dental analgesics and have been used as local anesthetics in dentistry since the seventeenth century, specifically to reduce pain associated with dental cavities [64].

Although most botanical bio-preservatives are used in foods that have been consumed safely for thousands of years, the present concern about their safety and use has subjected

products derived from them to extensive toxicological scrutiny [73]. Some EOs have shown toxicity, particularly to vulnerable people like the very old, the very young, and pregnant women [74]. This toxicity in essential oils may not only be effective when consumed internally but also when applied externally (or inhaled). For instance, evidence of chromosomal aberrations were found after exposure to eugenol [74]. Toxic reactions can be felt immediately, and range from dizziness, nausea, and allergic reactions to exhaustion, epilepsy and even death [75]. Eugenol may cause possible damage to the liver, where symptoms include, convulsions, diarrhea, nausea, rapid heartbeat and allergic reactions in humans [74,75]. The toxic effects of thyme are attributed to terpene phenols and data on Oral LD50 values (the lethal dose to 50% of animals in mg/kg of body weight) tested in rats can be used as an indication of toxicity. The Oral LD50 values of herbal parts or EOs of thyme and oregano are in the range of 1000-5000 mg/kg (see Table 2.1), while those for thymol and carvacrol are 980 and 810, respectively [65,76, 77].

Table 2.1 Lethal dose (LD50) for some essential oils in rats [Modified from 78]

Plant / herb	**LD_{50} (g/kg)**
Syzygium aromaticum (clove)	1–5
Thymus vulgaris (thyme)	2–5
Cinnamomum cassia (Chinese cinnamon)	2–5
Menta viridis (mint)	2–5
Daucus carota (carrot)	>5
Salvia officinalis (sage)	2–5
Rosemarinus officinalis (rosemary)	>5

The essential oils can still be used safely but the proportions of essential oils recommended must be respected, for example, an upper limit for inclusion of thymol established by the WHO is 50 mg/kg in food and 10 mg/L in beverages, where the residue of these compounds does not exceed the recommended levels [76,79].

Antimicrobial Activity

EOs and/or extracts plants have been selected as AM agents for various packaging materials; and the benefits of using them to reduce problems with contamination often caused by food production, distribution and safety are considerable. Many studies have emphasized that EOs and/or extracts plants of thyme and clove possess high antimicrobial activity against both Gram-positive and Gram-negative bacteria but the EOs and their compounds are slightly more effective against Gram-positive than Gram-negative bacteria (see Table 2.2) [80]. The inhibitory effectiveness of EOs is primarily due to the most abundant components in the EO; the association between the chemical composition of these components, their concentration and the AM efficacy in the EOs [73,81,82]. The growth of microorganisms inhibited by EOs is generally attributed to the presence of an aromatic nucleus containing a polar functional group [18,57,73,81], but other factors such as the hydrophilic/lipophilic balance are also likely to contribute to the extent of the growth [81]. The EOs and their components often affect multiple constituents; in particular, the membrane and cytoplasm, and in certain situations, they can alter the morphology of the cell [83].

Table 2.2 Antimicrobial effectiveness of EOs and their compounds [Modified from 84]

Organism	Type of AM agent	Degree of inhibition	References
Gram-negative bacteria	EOs	oregano > clove > thyme	Burt and Reinders 2003 [79]
	active constituents	eugenol > thymol / carvacrol	Friedman et al. 2002[76]
Gram-positive bacteria	EOs	thyme > oregano > clove / black pepper	Burt and Reinders 2003[79]
	active constituents	eugenol > linalool / allyisothiosyanate	Yamazaki et al. 2004 [80]
Yeast and moulds	EOs	thyme > clove/ rosemary/sage/bay	Farag et al. 1989 [75]
	active constituents	thymol / carvacrol > eugenol	Farag et al. 1989 [75]

Activity of Essential Oils against Bacteria

Gram-positive and Gram-negative bacteria have similar internal structures but extremely different external structures. Before studying the effects of EOs and their components on bacteria, it is necessary to understand the differing structures of the cell walls of Gram-positive and Gram-negative bacteria [83,84,85]. The cell wall of Gram-positive bacteria is made up of approximately 90%–95% peptidoglycan, other molecules; such as teichoic acid lipoteichoic acids proteins and complex polysaccharides (C polysaccharides) that are linked (Figure 2.1) [83].

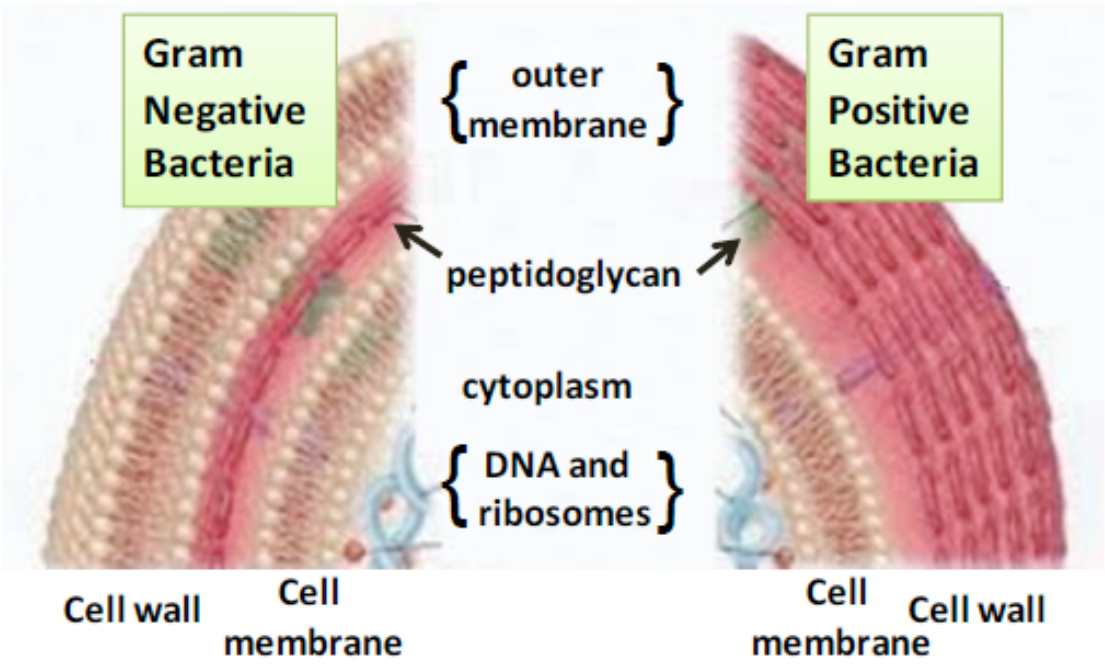

Figure 2.1 Gram-positive (on the right) and Gram-negative bacteria (on the left) [Adapted from 83]

The cell wall of the Gram-positive bacteria generally allows for the diffusion of hydrophobic molecules to easily penetrate the cells, and act within the cytoplasm and on the cell wall. The EOs containing phenolic compounds that exhibit antimicrobial activity against Gram-positive bacteria and their effect depends on the amount of the compound present. That means, at low concentrations they may interfere with enzymes involved in the production of energy, and at higher concentrations, they can denature the proteins [86,87].

The cell wall structure of Gram-negative bacteria is more complex than Gram-positive bacteria [88,89]. A Gram-negative cell wall consists of two external layers surrounding the cytoplasmic membrane. Immediately external to the cytoplasmic membrane is the peptidoglycan layer, which is thinner than the cell wall of Gram-positive bacteria and comprises of about 20% of the dry weight of the cell. In the Gram-negative cell wall there are no teichoic or lipoteichoic acids [88,89]. External to the peptidoglycan layer is the outer membrane (OM), which is singular in Gram-negative bacteria [88,89]. The peptidoglycan layer is covered by an OM that is primarily contained of amphipathic molecules (meaning that it has both hydrophobic and hydrophilic ends) called lipopolysaccharides (LPS) as well as various proteins. LPS composed of lipid A, the core polysaccharide, and the O-side (O-antigen) chain, which provides the impetus that allows Gram-negative bacteria to be more resistant to EOs and other natural extracts with antimicrobial properties [88]. The OM works as a protective barrier against macromolecules and hydrophobic compounds, which is why Gram-negative bacteria are relatively resistant to hydrophobic antibiotics and toxic drugs [88]. Nevertheless, small hydrophilic solutes can slowly traverse through porin proteins providing hydrophilic trans-membrane channels because the OM is not completely impermeable to hydrophobic molecules [89,90].

Indeed, the EOs and their compounds could cause a sudden significant reduction in viability of bacteria, if a critical concentration is applied. The permeability of the cell membrane is dependent on the hydrophobicity of solutes and the composition of the membrane [91].

Generally, the mode of action of EOs and their compounds have been not studied in great detail, owing to the large number of different groups of chemical compounds present in EOs, where it is crucial that the mode of action should be studied in multiple strains and species of microorganisms [90]. The mechanisms of action of the AM agents in EOs include the degradation of the cell wall [92, 93], coagulation of the cytoplasm [94,95,96], damage to cytoplasmic membrane and membrane proteins, increased membrane permeability leading to leakage of the cell contents [96,97], reduction of the proton motive force [98], and changes in the intracellular adenosine triphosphate (ATP) pool via decreased ATP synthesis and augmented hydrolysis that is separate from the increased membrane permeability and reducing the

membrane potential [98] (Figure 2.2). For example, carvacrol, an active component in many essential oils causes a reduction in the pH gradient and electrical potential in the proton motive force of the cytoplasmic membrane, as it has known to destabilize the cytoplasm and OM [93]. The collapse of the proton motive force and the degeneration of the ATP pool eventually lead to cell death. In this context, the following is known about the mode of action of thymol and eugenol [93].

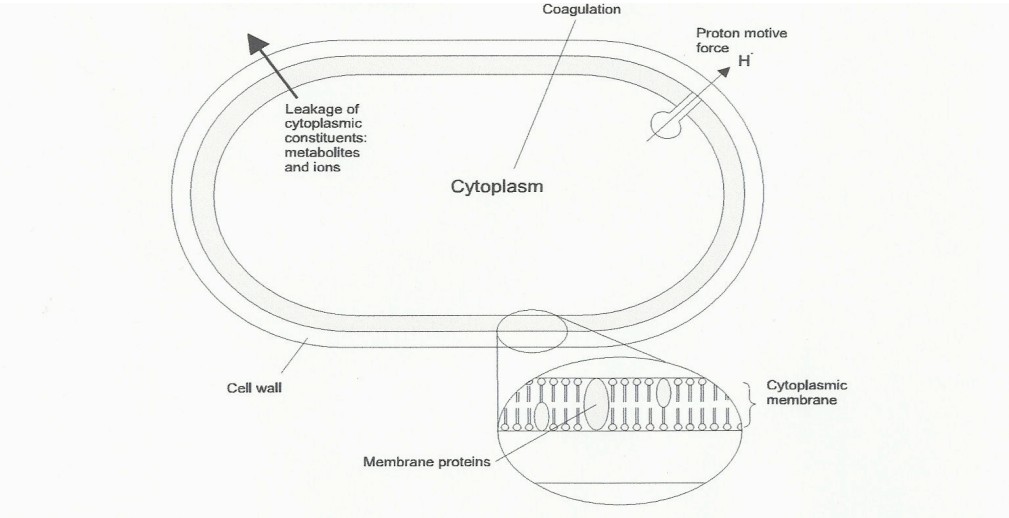

Figure 2.2 Sites in a bacterium where natural compounds are active [Adapted from 58]

Mechanisms of action

2.2.2.1 Thymol

As previously stated, the mechanisms of action of EOs and/or their components are dependent on their chemical composition. Thymol and carvacrol have different mechanisms of action against Gram-positive and Gram-negative bacteria, in contrast they have almost the same antimicrobial effects [91]. The antimicrobial activity is affected by the locations of one or more functional groups in these molecules [91]. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different position on the phenolic ring, but these differences do not affect the activity of either AM agent (Figure 2.3) [91]. The mode of action of natural

phenolic compounds like thymol and carvacrol, are known to cause structural and functional damage to the cytoplasmic membrane [96].

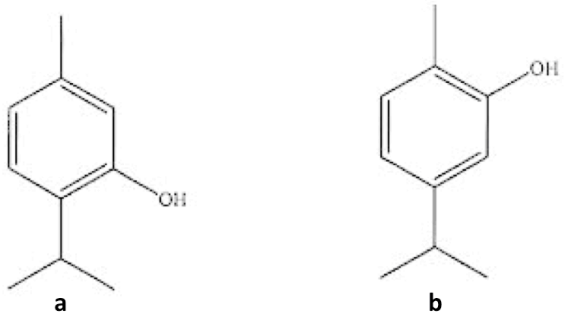

Figure 2.3 Structure of (a) thymol and (b) carvacrol

Thymol is a phenolic monoterpenoid and one of the major constituents of thyme oil and the antimicrobial action of thymol has received much attention in current research. Though the primary mode of AM action of the thymol compound remains obscure, it is thought to disrupt both outer- and inner- membranes and interact with membrane proteins and intracellular components. Prior research has indicated that in Gram-positive bacteria, thymol interacts with cell membranes and influences membrane permeability; resulting in loss of membrane potential, cellular uptake of ethidium bromide, and leakage of potassium ions, ATP, and carboxyfluorescein [92, 96, 99].

In Gram-negative bacteria, thymol is able to dismantle the OM of bacteria, leading to the increased the permeability of the cytoplasmic membrane [57,91]. As previously mentioned, the cell wall structure of Gram-negative bacteria is more complex than Gram-positive bacteria, thus, the permeability of the cell membrane is dependent on the hydrophobicity of solutes and the membrane composition [61,92]. Indeed, the reduced activity of these compounds against Gram-negative bacteria may be due to the presence of an obstacle that prevents the diffusion of phenolic components through the outer cell membrane. However, the hydrophobic component of the thyme oil (thymol) is still able to access the periplasm of Gram-negative bacteria through the porin protein [92]. After penetration into the inner part of the cytoplasmic membrane, the AM agents can cross react with the membrane proteins through the hydrogen bonds and hydrophobic interactions can occur causing a discharge of protons through the membrane, thereby affecting cellular activities governed by the driving forces of the protons [84].

2.2.2.2 Eugenol

Eugenol is a phenylpropanoid and one of the major constituents of clove oil. There are few EOs with phenylpropenes compounds and they can be found in eugenol, isoeugenol, vanillin, safrole and cinnamaldehyde [100,101]. Studies indicate that the free hydroxyl groups in these molecules are important for their AM activity against bacteria; where the AM activity of eugenol also might be attributed to the double bond in the α, β positions of the side chain, and a methyl group in the γ position [100] (see Figure 2.4). Moreover, the AM activity of the phenylpropenes also depends on the type and number of substitutions on the aromatic ring and, similarly to most other EOs, on the microbial strain and conditions in which the EO is tested like; choice of growth medium, temperature, etc. [102].

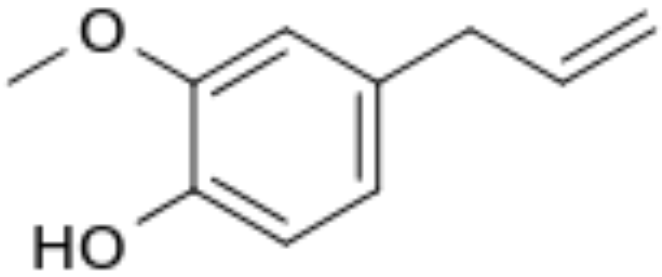

Figure 2.4 Structure of eugenol

Eugenol has an uncommonly higher antimicrobial activity against Gram-negative bacteria, yeasts, and molds than Gram-positive bacteria [61]. This is unusual for essential oil constituents because they generally are more effective against Gram-positive bacteria [61]. This could be attributed to the differing compositions of the OM of Gram-negative bacteria and Gram-positive bacteria. As previously indicated, the major components of the OM of Gram-negative bacteria are lipopolysaccharides (LPS), lipids, and proteins [83,103]. The Gram-negative bacteria have a thinner peptidoglycan layer, so eugenol can interact with the cell envelope readily. As Gram-positive bacteria do not have an LPS layer, this decreases the affinity of the antimicrobial system of euegnol and the bacterial interface [83].

The mode of action of eugenol is related to its ability to penetrate the cell membrane and interact with its proteins. Eugenol's action on membranes takes place primarily by a non-specific permeabilization of the cytoplasmic membrane, which has been described in several studies as the increased transfer of potassium and ATP out of the cell [93,99]. Eugenol alters the fatty acid profile of certain bacteria like, *Pseudomonas fluorescens*, *E. coli*, *Brochotrix thermosphacta*, *S. enterica*, and *S. aureus*, and causes cell damage to *E. coli* and *B. thermosphacta* cells [104,105]. The hydroxyl group of eugenol is thought to bind and affect the properties of proteins, thereby contributing to its inhibitory effect at sub-lethal concentrations. Correspondingly, eugenol has proven to inhibit the activity of the following enzymes: ATPase, histidine decarboxylase, amylase, and protease [101,106]. Inhibition of the ATPase may be important for cell destruction at high eugenol concentrations because energy needed for cell recovery is obstructed [101].

Antimicrobial Activity of Thyme oils, Clove oils and their compounds

The AM activity of plant-derived compounds against numerous various pathogens, tested individually and in vitro, is well documented in the literature [18,19,41,73,93,94,98]. However, the results reported in different studies are not easy to compare directly especially as contradictory results have been published by different authors about the same antimicrobial compound [107,108]. Accordingly, the three principal factors that can influence the outcome of the antimicrobial tests when EOs of plants and their compounds are used; the composition of the sample tested (type of plant, geographical location and time of the year), the microorganism (strain, conditions of growth, inoculum size, etc.), and the method used for growing and enumerating the surviving bacteria [109]. In vitro, the studies show that thyme and clove oils and their extracts have a stronger AM activity against spoilage, pathogens, molds and yeasts [57]. Dorman and Deans (2000), evaluated the antibacterial activity of thyme, clove and other medicinal plants against twenty-five different genera of bacteria including animal and plant pathogens, as well as bacteria causing food poisoning and spoilage using Iso-sensitest agar [57]. They discovered that all the organisms tested against clove and thyme to be sensitive, with thyme exhibiting more of an antibacterial effect than clove [57]. Prabuseenivasan et al. (2006),

evaluated the antibacterial activity of twenty-one plant essential oils like clove, orange, rosemary, lime, basil, thyme, cinnamon and others, against four Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Proteus vulgaris*) and two Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* at four different concentrations (1:1, 1:5, 1:10 and 1:20) using the disc diffusion method [110]. They found that both Gram-positive and Gram-negative bacteria were sensitive to the potency of the essential oils. *P. aeruginosa* and *P. vulgaris* were inhibited by nineteen oils, followed by *B. subtilis* (eighteen oils), *S. aureus* (fourteen oils), *E. coli* (twelve oils) and *K. pneumoniae* (nine oils) where the clove, cinnamon, and rosemary oils had strong antibacterial activity effects against all the organisms which were tested [109]. These two studies clearly signify that the importance of method, microorganism and composition can display diverse results.

Moreover, essential oils are rapidly being recognized as one of the most promising groups of natural compounds that can be developed for antifungal agents that are both safe and effective. Thyme oil, clove oil and their compounds have been reported to inhibit the growth of toxigenic fungal species, including the species of Aspergillius, Fusarium and Penicillium, and other non-toxigenic species, these compounds also effectively inhibit mycelium growth, [81,111,112,113,114]. About twenty-two extracts have recorded high antifungal activity against food-associated fungi and it was found that clove and ginger are more effective than other plant extracts, [115]. Clove oil and its compound, eugenol, has been widely used to limit mycotoxigenic fungi and mycotoxins [116].

In general, in vitro experiments do not necessarily provide a good indication of the potential value in food preservation. However, the results suggest that use of these compounds in foods could be effective but as research indicates, also may explain the reductions in AM activity of some EOs when applied to food products compared with in vitro performance [84,117,118]. To obtain effective AM benefits in food products, the required doses for EOs (or their constituents) may exceed organoleptically acceptable levels; as they are employed in higher doses than amounts used in flavoring applications, which might produce adverse sensorial effects. [117,118]. For instance, carvacrol is capable of retarding the growth of *Bacillus cereus (B. cereus)* in soup, but at an about 50-fold higher concentration than needed to achieve the same

effect as in a broth culture [119]. Therefore, there is an increasing request for correct and precise knowledge of the MIC of EOs and their compounds to enable a balance between the sensory acceptability and AM efficacy [96,120]. The MICs are defined as the lowest concentration of an AM agent (in mg/L or mg/ml) that will inhibit the visible growth of a microorganism on series of agar plates (agar dilution) or in broth (broth dilution) within a defined period of time [96,121] (see Table 2.3 + Table 2.4). The MIC is broadly used to evaluate the antimicrobial activity of EOs and their compounds but slight differences of their MIC data have been found owing to differences in test methodologies and in the criteria selected for the determination of the end-point. Therefore, the ability of comparing data from different studies is greatly limited [121]. For instance, Carson et al. (1995) describe the MIC as the lowest concentration of an AM agent that resulted in preventing visible growth of microorganisms over a 24 h contact time while Remmal et al. (1993) apply a similar definition of the MIC but extend the incubation time from 24 h to 28 h [121,122

Table 2.3 MICs of some EOs (mg/ml) [Adapted from 110]

Oils	***S. aureus***	***B. subtilis***	***K. pneumoniae***	***P. vulgaris***	***P. aeruginosa***	***E. coli***
Cinnamon oil	3.2	>1.6	3.2	>1.6	>0.8	>1.6
Clove oil	>6.4	>3.2	>6.4	>3.2	>1.6	>1.6
Lemon oil	>12.8	>12.8	>12.8	>6.4	12.8	>6.4
Lime oil	>12.8	>6.4	>6.4	>3.2	>6.4	>6.4
Oregano oil	>12.8	>12.8	12.8	>6.4	>12.8	>12.8
Rosemary oil	>12.8	>6.4	>12.8	>6.4	>6.4	>6.4

Table 2.4 MICs of some EOs components [Modified from 61]

Compound (Plant origin)	Chemical classification	Model organisms and measured minimum inhibitory concentrations (MIC)					Reference
		Gram-negative		Gram-positive			
		E. coli	*S. typhimurium*	*S. aureus*	*L. monocytogenes*	*B. cereus*	
Thymol (thyme)	**Monoterpenoid phenol**	225-5000 mg/mL	56.25-150 µg/mL	225-310 µg/mL	250 µg/mL	450 µg/mL	Cosentino et al. (1999) [73], Cristani et at. (2007) [124]; Rao et al. (2010) [125]. Ahmad et al. (2011) [126].
Carvacrol (oregano and thyme)	**Monoterpenoid phenol**	225-2500 µg/mL	150-250 µg/mL	450-1250 µg/mL	450-1500 µg/mL	900 µg/mL	Cosentino et al. (1999) [73], Kim et al. (1995) [123], Rao et al. (2010) [125].
Eugenol (clove)	**Phenylpropene phenol**	800-3000 µg/mL	3.18-500 µg/mL	2.12-750 µg/mL	800 µg/mL to a1000 µg/mL	n.a µg/mL	Gill and Holley (2004,2006) [101,102], Kim et al. (1995) [123].
Cinnamalde -hyde (cinnamon)	**Phenylpropene aldehyde**	397-1322 µg/mL	397 µg/mL	2.1-750 µg/mL	3965 µg/mL	0.3 µg/mL	Gill and Holley (2004,2006)[101,102], Hemaiswarya et al. (2011) [127].
p-Cymene (oregano and thyme)	**Monoterpene**	2500 µg/mL	n.a µg/mL	1250 µg/mL	n.a µg/mL	n.a µg/mL	Burt et al. (2007) [58], Ultee and others (2000) [119], Cristani et at. (2007) [124].
γ-Terpinene (oregano and thyme)	**Monoterpene**	5000 µg/mL	n.a µg/mL	2500–34000 µg/mL	n.a µg/mL	n.a µg/mL	Cristani et at. (2007) [124].

n.a. not analyses

Many studies have indicated that AM agents like EOs and their compounds when incorporated into films and coatings could be effective in inhibiting the growth of pathogenic microorganisms. Sanla-Ead et al. (2012) evaluated the antimicrobial activity of cinnamaldehyde and eugenol against three strands of yeast, eleven pathogenic and spoilage bacteria, where they determined the MIC of these compounds [128]. Then, they studied the antimicrobial effectiveness of cellulose-based packaging films containing the concentration of 1.0% w/w cinnamaldehyde and eugenol [128]. The experimental results showed that cinnamaldehyde and eugenol showed strong antimicrobial activity inhibiting the growth of all test strains but cinnamaldehyde showed lower MIC values against all test strains when compared with eugenol, whereas cellulose-based films containing cinnamaldehyde or eugenol completely failed to exhibit

a clear inhibitory zone in the presence of microbial growth around the film disc, this is attributed to the low water solubility of cinnamaldehyde and eugenol, and their low concentrations in the AM films [128]. The activation of Low Density Polyethylene (LDPE) films with thyme and oregano oils was carried out by using two different methods [129]. The LDPE films were prepared using the ionizing treatment (23±3 μm thick) and the LDPE films were incorporated using the extrusion method (27.5 ± 3.5 μm thick) [129]. The results demonstrate that the extrusion method showed a significantly higher inhibition against *E. coli* 0157:H7, *S. typhimurium*, and *L. monocytogene* than films developed using the ionizing method, which suggests that the extrusion method allowed a better incorporation of the active compounds on to the polymer [129]. However, there is limited information in using essential oils in plants as antimicrobials incorporated into films and coatings.

Applications in Food

There have not been many studies of the AM action of EOs and their compounds in model food systems and in real food owing to the greater efficacy of EOs in vitro when compared in real food [130,131,132]. For instance, the mint oil reduced the growth of *Listeria monocytogenes* and *Salmonella enteritidis* in culture media for two days at 30 ºC. Nevertheless, the activity of mint oil in paté (pH 6.8), and the traditional Greek appetizers tzatziki (pH 4.5), and taramasalata (pH 5.0) at 4 ºC and 10 ºC against these two bacteria was variable. *S. enteritidis* in all test conditions died off but not when inoculated in paté (pH 6.8) at 10 ºC. Similarly, *L. monocytogene*s numbers declined in the appetizers but increased in paté [133,134,135]. On the other hand, the antimicrobial efficacy of plant essential oils can be affected by the composition of food, like, the kind of oil or fat present in it. This was conspicuous when the efficiency of four plant EOs (clove, bay, thyme, and cinnamon) was evaluated in low-fat and full-fat soft cheese against *L. monocytogenes* and *S. enteritidis* at 4 ºC and 10 ºC, respectively, over a fortnight. In the low-fat cheese, all four oils at 1% reduced *L. monocytogenes* to below the detection limit of the plating method. In contrast, the oil of clove was the only substance to achieve such reduction in the full-fat cheese. In the full-fat cheese, the thyme oil was ineffective against *S. enteritidis*, despite the fact that this microorganism was completely inhibited in the broth culture [78]. Thyme oil was as effective as the other three oils in the low-fat cheese, reducing *S. enteritidis* to

less than 1 log CFU/g from day 4 onwards [118]. It was observed that the reduction in viable cell counts for pathogens in foods, which were tested, and their death rate depended on the pH, the storage temperature and the concentration of the EOs [136,137,138,139]. Microorganisms are more susceptible to the mechanisms of the AM agent during temperatures favorable to their optimum growth potential because of their increased metabolic activity under these conditions. According to Hao et al. (1998), an increased storage temperature enhances the efficacy of some plant-derived compounds like carvacrol, against food-borne pathogens such as *L. monocytogenes* and *A. hydrophilla*. It was found that the temperatures during incubation and exposure at 8 °C and 30 °C had a significant influence on the survival of vegetative cells *of B. cereus*, with a higher death rate of B. cereus at 30°C [140]. It was concluded that carvacrol migrates more easily into the membrane of *B. cereus* because of its higher membrane fluidity at 30 °C compared at 8 °C. In another study, it was also observed that the rate of death in Shigella in fruit juices is higher at room temperature compared with refrigeration [141]. Consequently, using herbal plant or spices and their extracts on food packaging materials can provide good protection against temperature abuse during storage and distribution.

The pH value of the food medium is believed to have a strong effect on the AM activity of the EO. It was observed a stronger AM influence in thymol against *S. typhimurium* at pH 5.5 compared with pH 6.5. At low pH levels, the thymol molecule is mostly binding, more hydrophobic, where it may bind better to hydrophobic regions of membrane proteins and dissolve better in the lipid phase of bacterial membrane [142]. It has also been reported that the AM activity of carvacrol increased against *B. cereus* at acidic pH 5.5 compared to neutral pH 7.0 [143]. Shaik et al. (2005), performed a study to determine the in vitro effects of eugenol and cinnamaldehyde against thirty-one strains (twenty-nine indigenous, one standard strain of Helicobacter pylori ATCC 26695, and one strain of E. coli NCIM 2089) at three different concentrations (1µg/ml, 2µg/ml and 4µg/ml), different incubation hours and at various acidic pH levels (pH 4, pH 5.2 and pH 7) [144]. The results showed that at a concentration of 2 µg/ml in the 9th and 12th hours of incubation respectively, and acidic pH (pH 4.0); both the compounds inhibited the growth of all the thirty *H. pylori* strains. At acidic pH for both compounds the AM activity increased, with a higher AM activity of both compounds at acidic pH 4.0 [144]. The

activity of these compounds at low pH levels may help EOs and their compounds achieve their effectiveness and correspondingly, the inhibitory activity should decrease at high pH levels [143].

As previous studies have indicated, the AM activity of EOs and their compounds demonstrated in vitro might not be a good indication of practical value in food preservation as it has been established that microorganisms in food are less susceptible to EOs and their compounds than in vitro. Furthermore, external factors like temperature also affect the antimicrobial effectiveness of the essential oils [131].

Additionally, the active compounds of essential oils bind with food components; e.g. fats, sugars, salts, etc., and may compromise their effectiveness. For example, only a proportion of the EO added to the food will exhibit AM activity as some of the EOs and their compounds are subject to neutralization, or rapid diffusion when applied to the food [58]. These examples also highlight the need for future research into appropriate modes of application of EOs and their compounds.

Originality

The literature review has demonstrated that herbal plants and spices are excellent and rich sources of antimicrobial agents that can act against various microorganisms; for e.g. bacteria, yeasts, molds, etc., that degrade and deteriorate food. Using these natural AM agents in packaging materials, provides food with adequate protection from the growth of pathogenic organisms. Under appropriate conditions, the use of EOs and their compounds in food packaging materials helps to control microbial growth, increase food safety, and extend product shelf life [18]. Even though, all previous studies were concerned about the use of extracts and/or EOs as AM agents, but not the ground plants themselves, and to our knowledge, there are no studies that have been conducted by comparing the antimicrobial performance of the original ground plant powders versus EOs directly applied into films or fibers. On the other hand, some EOs have shown toxicity, particularly to vulnerable people like the very old, the very young, and pregnant women, as previously mentioned [74]. Moreover, EOs can evaporate during processing and blending at high temperatures and applications at room temperatures, which can affect their efficiency. Furthermore, deriving an EO is an expensive and laborious process when compared with the process of obtaining ground plants and spices.

Therefore, the originality of the project is to appraise the inhibitory activity of ground plant of clove bud powder against specific microorganisms that represent a potential spoilage risk in the food. This study evaluates and compares the efficacy of a range of various ground, powdered plants as antimicrobial agents. In addition, for each tested bacteria, the MICs and MBCs were also quantified. Even though many studies have analyzed the antimicrobial activity of EOs and their compounds when incorporated into films and coatings, none have studied the antimicrobial effects after the incorporation of clove bud powder on/ into polymers.

Objectives

General objective

Upon reflecting the current demands and research in the area of AM food packaging, this study is aimed at developing antibacterial food packaging based on ground plants like the powdered clove bud. To achieve this objective, the following specific objectives have been considered.

Specific objectives

- To examine the antimicrobial activity of clove bud and its ability to stop or inhibit the growth of one Gram-negative bacteria *E. coli* and two Gram-positive bacteria *L. innocua* and *S. aureus*.
- To determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of clove bud powder against *E. coli*, *L. innocua* and *S. aureus* bacteria.
- To investigate the potential use of clove bud powder as an AM agent on packaging films and fibers using LDPE and PCL respectively, as polymeric substrates.

CHAPTER 3 MATERIALS AND METHODOLOGY

Materials

Polymers

The polymers used to prepare the films and fibers for the present study were low-density polyethylene Resin (ExxonMobil™ LDPE LD 100.BW) and Linear Low Density Polyethylene Resin (ExxonMobil™ LLDPE LL 3003) and poly (ε-caprolactone) (PCL) CAPA® 6800 (Perstorp Winning Formulas) the characteristic properties of the polymers are presented in Table 3.1.

Table 3.1 Characteristic properties of the polymers used to prepare the AM films and fibers

Materials	Density (g/cm^3)	Melt flow index (MFI) (190°C/2,16 Kg)	Melting point (Tm °C)	Suppliers
LD100BW	0.923	2	110 (140-170)*	Exxon Mobil™
LL 3003	0.923	3.2	124 (278-305)*	Exxon Mobil™
PCL CAPA® 6800	1.14	7.29	60-62 (70 -120)*	Perstorp Winning Formulas

* Extrusion temperature conditions or * extrusion melting conditions

Antimicrobial Additives (AM)

The AM additives used in this experiment was thymol powder (obtained from Wintersun Chemical) and ground plants which included; sage, clove bud, clove leaf, lemongrass, black mustard seed, yellow mustard seed, wild mint leaf, and thyme leaf purchased from local stores in Montreal, Quebec, Canada. Additional details of the thymol powder as an AM additive is presented in Table 3.2.

Table 3.2 Product characteristics and properties of the AM agent thymol

CAS Number	**89-83-8**
Molecular Formula	2-[$(CH_3)_2CH$]C_6H_3-5-(CH_3)OH
Molecular Weight (g/mole)	150.22
Density at 20 °C (Kg/ m^3)	980
Appearance/Physical State	White crystals to powder
Assay	99% Min

Film Production and Blend Preparations

Production of Film by Extrusion

Film grade LD100BW resin pellets were blended with 2% thymol powder using a Twin Screw Extruder (Leistritz ZSE-18HP-40D, Germany) with a screw rotation speed of 119 rpm (rotations per minute). The die is maintained at a temperature of 195 °C, however, the actual temperature recorded at the end of the outlet of the die is much less than 195 °C. This is due to the continuous stream of air having a flow rate of 25 KPa and from the cooling nozzles, placed very close to the die exit. The film that was produced had a thickness of 140 μm with an average of five readings taken at different points on the film sample. The profile temperature that was used is shown in Table 3.3. After cooling, white powder started to form onto the surface of the film. This film was used to study the migration kinetics of thymol from LDPE to the surface of the film over time by using Fourier Transform Infrared Spectrometer FTIR–ATR spectroscopy test.

Table 3.3 Temperature profiles in twin-screw extruders

Zone	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Die**
Temperatures (°C)	160	170	170	175	175	180	180	190	195

Production of Sheets Film by Brabender

Film grade LL3003 resin pellets and 2% of thymol were blended directly and mixed to ensure uniformity using a Brabender internal mixer GmbH & Co. KG (Duisburg, Germany), and the volume of the mixing chamber was 30 cm^3, where the mixing time was eight min., the speed rotation was 100 rpm, and the temperature used was around 190 °C. The mixing time allowed stabilization of the fixed temperature and torque. Thereafter, the samples with 2% of thymol were pressed into sheet of 150 μm using a hot press at 190 °C for six min. By using the internal mixer for the preparation of the samples, an improvement in the quality of the mixture was noted due to the absence of crystals on the surface of thymol films. Also these sheets were perforated to study the migration kinetics of thymol from the LLDPE to the surface of the film over time by using FTIR–ATR spectroscopy test.

Production of fibers by the Electrospinning Technique

In a mixture of dichloromethane and dimethylformamide (obtained from the Sigma Chemical Company) (DCM: DMF) (50:50) v/v 15% of PCL and 200 mg of thymol was dissolved for about 24 h. Dissolution was carried out under magnetic stirring at room temperature until complete dissolution of the solutes. The PCL polymer solution was drawn into a 3 ml plastic syringe and the needle tip at its center was connected to a high-voltage power supply by an alligator clip. The voltage applied between the needle tip and the collector is in the range of 16-18 kV (see Table 3.4). When the electric charge of the polymer solution at the needle tip is greater than the forces of surface tension and viscosity a polymer jet is ejected. The jet caused by the electric field will be directed towards the collector, the solvent evaporates and the spun nanofiber mats are collected on an aluminum foil [145,146]. Electrospun PCL nanofibers containing thymol were produced to investigate the antimicrobial activity.

Table 3.4 Electrospinning: processing parameters

Parameters	Value	Unit
Electrospinning distance	15	Cm
Diameter of needle	18 (0.838)	gauge (mm)
Flow rate	1	ml/h
Voltage	16–18	kV

Characterization

Thermal analysis

Thermal analysis comprises of a set of techniques that measure the response of the material being heated or cooled in order to establish the connection between temperature and physical properties. Thermogravimetric analysis (TGA) is one of these techniques. TGA. It is based on measuring the variation of the mass in a sample when it undergoes a temperature change in a controlled atmosphere. This variation may be a loss or gain of mass due to decomposition, oxidation, or loss of volatiles (such as moisture) [147]. This technique is used to determine the degradation temperature of thymol and the clove bud powder, and the actual amount of clove bud powder on PCL fibers and surface of LDPE film. The specimens were analyzed using a (TGA) Q5100 (TA Instruments, New-Castle, DE) and tested under nitrogen (N_2) atmosphere from 25 to 800 °C at rate of 10 °C/min.

Surface analysis

The Scanning Electron Microscope (SEM) is an instrument used for the observation and surface analysis of biological and mineral samples by providing information on topology, texture, size and shape, and chemical composition (EDS). The size and shape of clove bud powder is analyzed by the SEM (FESEM, Hitachi S-4700).

Particle size analysis

The particle size was determined with a Coulter LS200 Laser Granulometer. This technique allows grain-size analysis of particles suspended and transported in water. For each specimen, the numerical files displaying the complete distribution of grain size through histograms or cumulative curves are provided by the processing software, including the main granulometric parameters such as the mean grain size and the standard deviation.

Fourier transform infrared (FTIR)

An infrared spectrometer using the method of the Fourier transform was used to monitor the migration of the thymol powder to the surface of the film and sheets. The tests were performed using a Spotlight 200 FTIR instrument, equipped with a Germanium crystal ATR, from Perkin Elmer (Waltham, MA), with a resolution of 4 cm^{-1} and an accumulation of 16 scans. Following the acquisition of spectroscopic data, the Peak Fit V4 software was used to de-convolute each spectrum individually, which allows fitting into a Gaussian curve to each individual sub-peaks of a spectrum.

Antimicrobial Tests

Test in vitro

Antibacterial tests in vitro were carried out in the Department of Microbiology, Infectiology, and Immunology at the Université de Montréal. In the present study, two methods of antibacterial tests were used to investigate the effectiveness of the thymol and the plants as antimicrobial (AM) agents against the Gram-negative *Escherichia coli (E. coli)* (DH5α) and Gram-positive *Listeria innocua (L. inncoua)* (LSPQ3284) and *Staphylococcus aureus (S. aureus)* (54-73). The first method was used to study the antimicrobial activity for samples in the form of polymeric films containing antimicrobial agents, while the second method was used to study the

antibacterial properties of nanofibers containing the antimicrobial agents. The preliminary stage of preparation of the reagents and bacterial broth were common in both methods. The enumeration of colonies was used to determine the rate of reduction of bacterial growth resulting from the action of each antibacterial agent. The number of colony-forming units (CFU/ml) was determined by

$$CFU/ml = \frac{\text{number of colonies}}{\text{dilution*volume drop (ml)}}$$

The media culture was prepared in accordance with the manufacturers instructions, in 250 ml flasks.

3.4.1.1 Preparation of LB (Luria-Bertani) broth and agar

The LB (Luria-Bertani) medium is very commonly used to grow *E. coli* and other related enteric species. It contains tryptone, yeast extract and sodium chloride (NaCl). It was originally formulated by Giuseppe Bertani in 1951 for studying lysogeny in *Escherichia coli*. A 6.25 g of LB broth is dissolved in 250 ml of distilled water. This solution is stirred manually until the powder is dissolved completely and then sterilized in an autoclave for 45 min. The LB broth is used to culture the bacterial strain.

The LB agar is prepared in the same way by dissolving 10 g of LB agar in 250 ml of distilled water. The mixture is stirred manually until the powder dissolves completely and then sterilized in an autoclave for 45 min. The LB agar is then poured into Petri dishes and they can be stored at 4 °C. The LB agar is used to grow bacteria on solid media and can be used to count individual colonies.

3.4.1.2 Preparation of phosphate buffered saline PBS

PBS is a buffer solution primarily used in biological research. It helps to maintain a pH balance. It is a solution containing sodium phosphate and sodium chloride and in some

formulations, potassium chloride and potassium phosphate. $PBS_{(10X)}$ is diluted ten times by adding 225 ml of distilled water to 25 ml of $PBS_{(10X)}$ and sterilized in autoclave for 45 min. where it is then added 900 µl of PBS_{1X} to each eppendorf tube. PBS_{1X} solution will be used to re-suspend the bacteria and fiber samples, to wash the film samples and also to dilute the bacterial colonies in order to reach the desired concentrations in both samples.

Static method

In this method, the samples were formed into polymer films or sheets. In this case, the AM agents may be coated onto or incorporated into the polymer films or sheets. This method requires a dilution of bacterial broth. The initial inoculum, having spent a night in the incubator at 37 °C contains approximately 10^9 CFU/ml. Before conducting the antibacterial tests, this inoculum is diluted to a volume basis of 1 to 10 volumes, three successive times to reach a concentration of 10^6 CFU/ml.

A 100 µl from a culture broth concentration of 10^9 CFU/ml is then added to 5 ml of LB broth that results in a concentration of 10^8 CFU/ml. This procedure is repeated twice over to obtain a final concentration of 10^6 CFU/ml. Both active and blank samples are cut out into discs of diameter ranging from 4 to 5 cm. Standards require that the surface of contact between the two polymer layers to be between 400 and 1600 mm^2. Blank discs should be sterilized by washing with 70% ethanol before the test. The two discs (one blank and the other active) are sandwiched together after 200 µL of 10^6 CFU/ml culture is deposited between them. Both films are rubbed against each other, so the surfaces are contaminated. The films are placed in a Petri dish, and incubated at 37 °C for a period of 24 h. The next day, both discs are quenched in a test tube with 2 ml of pre-prepared saline solution (PBS_{X1}). Each sample is made in triplicate, along with the negative control containing two blank discs.

Dynamic method

Antibacterial tests are conducted on electrospun fiber mats produced with the

incorporation of AM agents. The dynamic method ensures a better contact between the bacteria and fibers treated by supplying constant agitation of the sample in a suspension during the test period. The fiber mats are cut into small squares, so that each weighs approximately 1g. Then 100 μL of bacterial culture, which has already been incubated for 24 h, is added to 100 ml of sterile PBS. Next, 5 ml of this solution is added into each test tube containing a fiber mat. This is also done in triplicate with a pure fiber sample and a negative sample of bacteria-only solution. The tubes are then placed in an incubator at 37 °C to shake for 24 h.

Counting bacteria

Both characterized methods mentioned above require dilution to assess the rate of bacterial growth provided for each sample. In the static method (films), the discs are placed in the wash solution of 2 ml of PBS. In the dynamic method (fibers) the mats will have spent the night in the incubator with 5 ml of PBS. Both films and fibers will experience successive dilutions and for each case, 100 μL from the final solution is added to a first eppendorf containing 900 μL of PBS, where the dilution is 10^{-1}. Then 100 μL of the medium is taken from the eppendorf 10^{-1}, and then added to a second eppendorf, which corresponds to a 10^{-2} dilution. This is continued in the same way for the 10^{-3} and 10^{-4} dilutions. Using a micropipette, three 10 μL samples are taken from each dilutions and droplets are applied on to the Petri dish containing the LB agar in triplicate. Then the Petri dishes are placed in an incubator at 37 °C for 24 h. The number of bacteria of each drop and the concentration of the sample was calculated as CFU/ml.

$$CFU/ml = \frac{\text{number of colonies}}{\text{dilution*volume drop (ml)}}$$

Determination of the minimum bactericidal concentration (MBC) without sterilization

The minimum inhibitory concentration (MBC) was determined for clove bud powder on an agar plate. In the testing tubes, a bacterial culture (approx. 10^6 CFU/ml) was inoculated into

LB broth in different concentrations of clove bud powder incubated at 37 °C with shaking for 24 h. After incubation, l00 µl was taken from each tube and spread across the surface of an agar plate to dry before incubation for 24 h at 37 °C. A blank control plate was prepared in exactly the same way without clove bud powder. All experiments were carried out in triplicate. The MBC of the clove bud powder was defined as the lowest concentration of clove bud powder at which no growth occurred on the plates.

Determination of minimum bactericidal concentration (MBC) using two different methods of sterilization

The main objective of this test was to determine if the same values of MBC for clove bud powder are obtained by using two different sterilisation methods. Sanitization is the process of using a physical or chemical process to remove, destroy, or inactivate all living organisms, spores, and endospores on a surface so that the organisms are no longer infectious [148]. In this study, the two physical methods used to sterilize the clove bud powder before performing the MBC test, were heated in an autoclave and ultraviolet (UV) radiated. The UV system used in testing was a Cole-Parmer Ultraviolet sterilizer lamp (Catalog No. 9762014). In the first method, the clove bud powder was prepared and sterilized in an autoclave at 121 °C for a period of 20 min and the procedure repeated as outlined above (Section 3.4.5). In the second method, the clove bud powder was prepared and sterilized under UV radiation lamp with 254 nm wavelength for 20 min and the procedure repeated as outlined above (Section 3.4.5). It has been determined that 254 nm UV light is the optimal wavelength to inactivate or destroy bacteria and spores [148].

CHAPTER 4 ORGANIZATION (PRESENTATION OF PAPERS)

The main outcomes of this research study are presented in the form of two scientific papers detailed in the next two chapters.

Chapter 5 presents the results of the first paper, *"Antibacterial Properties of Electrospun Fibers of PCL/Clove Bud Powder"* that was presented in the annual Technical Conference of the Society of Plastics Engineering, ANTEC 2014. In this paper, the work was focused on evaluating the antibacterial activity of selected powdered plant species such as sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf against pathogenic bacteria like *E. coli* (DH5 α), and determining the MBC values of clove bud powder after using different methods of sterilization. The antimicrobial capacity of the clove bud fibers was also determined against food borne bacteria and the amount of clove powder was determined in the PCL mat by using TGA.

Chapter 6 presents the second article, *"Development of Antibacterial Structures and Films Using Clove Bud Powder"* that was submitted to Industrial Crops and Products. In this work, the antimicrobial activity of clove bud powder was investigated against one Gram-negative bacteria, *E. coli* (DH5 α) and two Gram-positive bacteria, *L. innocua* (LSPQ3284) and *S. aureus* (54-73). The MIC and MBC values of the clove bud powder were determined, including the size of clove bud particles that was reduced and analyzed by using dry ring milling (Vibratory Disc Mill) and a Coulter LS200 Laser Granulometer in water, respectively. Furthermore, this study investigated the antimicrobial effects of clove bud powder when coated onto film LD100.WB matrixes at two different level/concentrations. Finally, the antimicrobial activity of the clove bud powder was investigated, when blended with PCL via the solution-casting technique.

CHAPTER 5

Antibacterial Properties of Electrospun Fibers of PCL / Clove Bud Powder [1]

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Abstract

The antimicrobial properties of essential oils another plant extracts have been known for many years and have been used against a wide variety of bacterial pathogens as well as several fungi. The purpose of this study is to investigate and compare the antimicrobial activities of various ground powdered plants such as sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf against *E. coli (DH5 α)*.

The clove bud powder showed the highest antimicrobial activity compared to the other ground plants used in this study. The minimum bactericidal concentration (MBC) of the clove bud powder was measured and then its antimicrobial activity was monitored for the electrospun PCL and clove bud powder blends dissolved in a mixture of (DCM: DMF) (50:50) v/v. The antimicrobial activity of the PCL and clove bud fibers was assessed using dynamic method.

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[1] **A part of this work was submitted to the 72nd annual Technical Conference of the Society of Plastics Engineering, ANTEC 2014 in Las Vegas, April 2014.**

Introduction

The demand for handling food and its safe consumption has intensified in recent years. The frequent incidences of food poisoning from contaminated food worldwide have heightened research and development in the field of food safety to improve human food storage and consumption [1]. These widespread contaminations could be attributed to a variety of factors, such as the increase in global trade, the export of food products, change in current methods of food production, changing modern lifestyles, changes in food consumption, and the emergence of new pathogens. Recent studies have investigated the use of plant extracts and essential oils in active packaging [2]. Using plant extracts or essential oils in the packaging materials under appropriate conditions prevents or limits many problems related to the growth of bacteria such as *Escherichia coli (E. coli)* [3]. The plant extracts and essential oils in packaging materials are used to reduce or eliminate food-borne microorganisms, thus extending the shelf life of food and improving safety standards [4].

Several studies have been conducted on plant extracts and essential oils as antimicrobial agents [5,6,7]. The antimicrobial properties of plant extracts and essentials oils in plants have been recognized for many years [8]. These extracts are obtained from different parts of plants and do not affect the flavor of the food nor its chemical properties [9,10,11]. Essential oils (also called volatile oils) are complex mixtures comprising of many single compounds. Chemically, these volatile oils are derived from terpenes and their oxygenated compounds. Each of these constituents contributes to the beneficial or adverse effects [2,4,12]. Correspondingly, the action mechanism of essential oils as antimicrobial agents in films and fibers have received significant attention from researchers [13,14,4]. Investigation on the antimicrobial effects of several plant powders revealed that the clove bud powder has the most effective antimicrobial activity. Clove is also known as *Syzygium aromaticum* and belongs to the myrtle family, Myrtaceae. The essential oil extracted from clove has many therapeutic effects, including kidney reinforcement, anti-vomiting, antispasmodic and anti-phlogistic. It is widely used in medicines, especially in the preparations for gum and teeth [15,16]. It is also used in the fragrance and flavoring industries [17,18]. In addition, the essential oil extracted from clove bud has biological properties such as

antibacterial, antifungal, insecticidal and antioxidant [17,18]. It is also used traditionally as flavoring agent and antimicrobial additive in food [19,18]. Some studies have reported that the leaf and buds oils were complex mixtures of numerous compounds; many of which were present in trace amounts [16, 18, 19]. Each of these compounds has different antimicrobial properties. However, many studies showed that the clove bud oil has high antimicrobial activity against both gram-positive and gram-negative bacteria [20]. The two major constituents in clove oil are eugenol and beta-caryophyllene where the high concentration of eugenol is responsible for the strong antimicrobial activity [15, 16, 18].

All the previous studies were concerned about the use of extracts and/or essential oils as antibacterial agents, but not the ground plants themselves. The direct use of powders is their ease of dispersion in plastic films or other packaging materials. In this study, the antimicrobial activity of clove bud powder was evaluated and compared to many other plant powders by determining its minimum bactericidal concentration (MBC) and its efficiency in electrospinning with PCL. No study has been conducted on comparing the antimicrobial performance of the original ground plants powders versus essential oils directly applied into films or fibers.

Electrospinning has attracted much attention over the past decade because of its high cost and low production rates. The electrospinning technique is a straightforward method to produce fibers from polymer solutions [21, 22]. The electrospun nanofibers may have a surface area twice as large as that provided by the thin and continuous films [21,22,23]. This large amount of surface area available is likely to provide improved properties and sensitivity of the material in several areas of application. A high voltage is used to produce a membrane composed of small-interconnected fibers with diameters in the wide ranges of 50 nm – 2000 nm [21,22]. The nanofiber fabrication with the electrospinning technique provides a very high specific surface area because of the small nanofiber diameters, which leads to highly porous membranes with excellent sticks of electrospun fibers, such as high porosity, small-diameter, excellent pore interconnectivity and high surface-to-volume ratio [25]. The above characteristics combined with the functionalities of the polymers supply nanofibers with specific properties important in advanced applications [22,25]. Taking into account that the advantages provided by the electrospinning method, it will be operated during this project to study the effect of the

concentration and particle size on the antibacterial activity of AM agents. The electrospinning device to be operated is a device consisting of a homemade brand pump and a Harvard Apparatus Model Gamma-5W ES6OP generator provided by Gamma High Voltage Research Inc.

In this study, electrospun poly (ε-caprolactone) PCL nanofibers containing clove bud powder were produced to investigate the antimicrobial activity of clove bud powder against *E. coli (*DH5 α*)*, which is one of the common food borne pathogenic bacteria.

More specifically, the objectives of this study were to evaluate the antimicrobial activity of powdered plants such as sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf on their ability to stop or inhibit the growth of foodborne pathogenic bacteria such as *E. coli.* The MBC, which is the lowest concentration of clove bud powder that can kill 99.9% of *E. coli* after a 24 h incubation period, was evaluated. Then, the antimicrobial activity of the plants powder was investigated in their blends with PCL electrospun fibers.

Materials and Methodology

Materials

The ground plants including sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf were purchased from the local stores in Montreal, Quebec, Canada.

Preparation of ground plants and antibacterial testing

The plants were dried and ground using an electric mill grinder. The antibacterial tests on the various ground plants were conducted in the Department of Microbiology, Immunology and Infectious Diseases at Université de Montréal. In separate tubes, 200mg of each powdered plant was mixed with a mixture of 5 ml *E. coli (DH5 α)* and Phosphate-Buffered Saline (PBS_{X1}) (approximately 10^6 CFU/ml). The experiment was carried out in triplicate. The tubes were then placed in the incubator at 37 ° C for 24 h. Before and after incubation, the pH value was ranged from 5 to 6 in all the tubes.

Determination of minimum bactericidal concentration (MBC)

The MBC was determined for clove bud powder on an agar plate. In the testing tubes, a bacterial culture (approx. 10^6 CFU/ml) was inoculated into Luria-Bertani (LB) broth in the presence of different concentrations of clove bud powder ranging from 20-200 mg/ml and incubated at 37 °C with shaking for 24 h. After incubation, l00µl was taken from each tube and spread across the surface of an agar plate to get dried before incubation for 24 h at 37 °C. A blank control plate was prepared in exactly the same way except that no clove bud powder was used. All experiments were carried out in triplicate. Before and after incubation, the pH value was measured in all the tubes obtaining pH results ranging from 5 to 6. These results are the optimal pH value for the growth of *E. coli* cells, which is usually around the neutral range of pH 7. On the other hand, the clove bud powder was then prepared and sterilized using two different methods as described in Section 3.5.6 in an autoclave at 121 °C for a period of 20 min and under UV Sterilization Lamp with 254 nm wavelength for 20 min and the procedure repeated as the test (Section 3.5.6). The MBC of clove bud powder was defined as the lowest concentration of clove bud powder at which no growth of *E. coli* occurred on the plates [3,20,26].

Preparation of the solution for electrospinning

10% (w/v) of poly (ε-caprolactone) (PCL) CAPA 6800 (obtained from Perstorp Winning Formulas) was dissolved in a mixture of dichloromethane and dimethylformamide (obtained from the Sigma Chemical Company) (DCM: DMF) (50:50) v/v, and stirred using a magnetic stirrer for approximately 24 h at room temperature. Then, 200 mg of clove bud powder was added to the PCL polymer solution and stirred using a magnetic stirrer for two hours. Electrospinning was carried out at room temperature and the process has been fully described in the literature [27,28]. PCL polymer solution was drawn into a 3 ml plastic syringe and the needle tip at its center was connected to a high-voltage power supply through an alligator clip. The applied voltage was in the range of 15-17 kV between the needle tip and the collector (see Table 5.1). When the electric charge of the polymer solution at the needle tip is greater than the forces of surface tension and viscosity a polymer jet is ejected. The jet caused by the electric field will

be directed towards the collector, the solvent evaporates and the spun nanofiber mats are collected on an aluminum foil. The parameters used in this case are presented in Table 5.1.

Table 5.1 Electrospinning: processing parameters

Parameters	Value	Unit
Electrospinning distance	15	cm
Diameter of needle	18 (0.838)	gauge (mm)
Flow rate	1	ml/h
Voltage	15–17	kV

Antibacterial tests were conducted on the electrospun fiber mats produced with incorporation of clove powder. The dynamic method ensures a better contact between the bacteria and fibers treated by supplying constant agitation fiber mats were cut into small squares, so that each weighed approximately 1g. 100 µL of bacterial broth, which has been already incubated for 24 h, was then added to 100 ml of sterile PBS_{X1}. 5 ml of this solution was added into each test tube containing a fiber mat. This was also done in triplicate with a pure fiber sample and a negative sample of bacteria-only solution also used. The tubes were then placed in an incubator at 37 °C to shake for 24 h.

Results

Antimicrobial activity of ground plants

The antimicrobial activity of sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf were tested using *E. coli (DH5 α)* bacteria and the results are shown in Fig. 5.1. It is clear that the clove bud powder killed all the bacteria, indicating that it has the best antibacterial activity compared to all other plant powders. Also, surprisingly, Fig. 5.1 shows that sage, lemongrass, and wild mint leaf have higher growth of *E. coli compared* with the control sample. This could be because the plant powder of those plants contains a mixture of compounds, which could increase bacterial growth. Clove leaf, black mustard seed, and thyme leaf showed some significant changes.

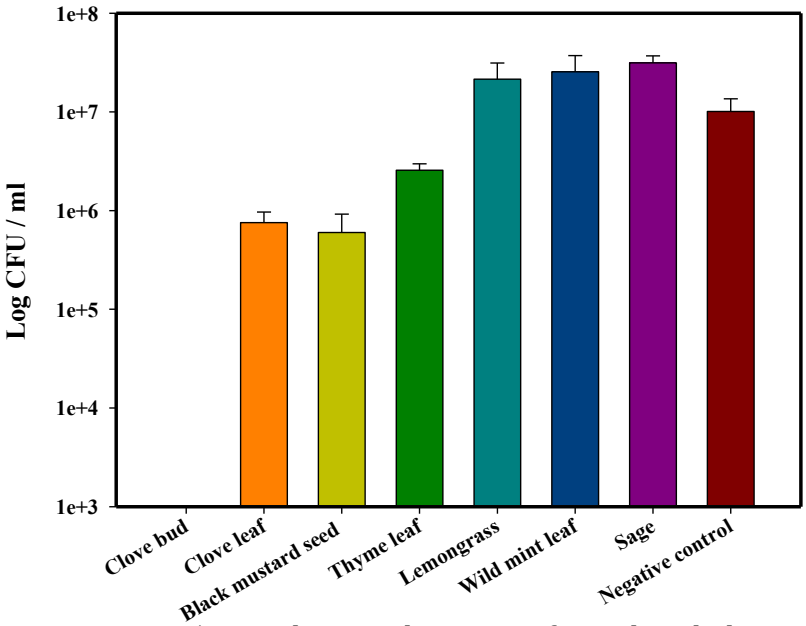

Figure 5.1 Antibacterial activity of powdered plants

Minimum bactericidal concentration (MBC) with/ without sterilization

The clove bud with sterilization showed lower MBCs against *E. coli* strain than those of clove bud without sterilization. The MBC without sterilization for clove bud powder at different concentrations (see Figure 5.2). The MBCs value of clove bud powder is about 200 mg/ml, implying that if the concentration is less than 200 mg/ml, then the bacteria will start to grow. The results of the MBC of clove bud powder with two different methods of sterilization were ≥ 50 mg/ml. This could be explained by the use of heat and UV light to sterilize the clove bud powder, it is possible to conclude that due to the heat applied some chemical compounds were activated.

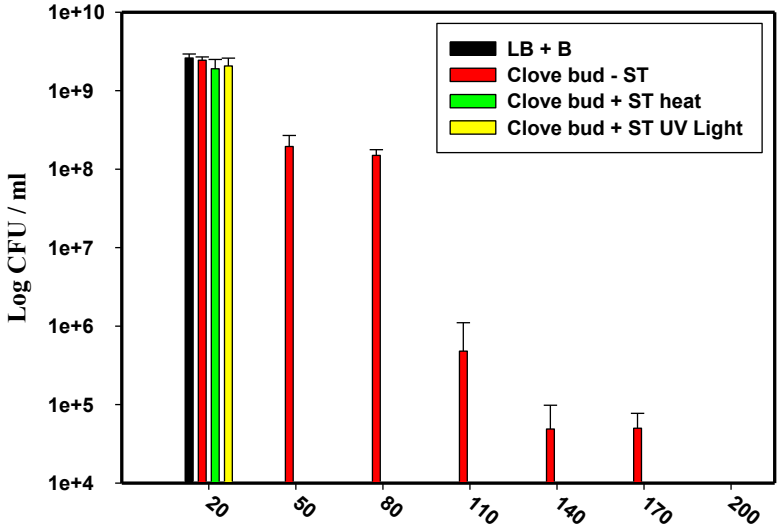

Figure 5.2 Minimum bactericidal concentration (MBC) of clove bud powder (mg/ml)

Antibacterial activity of PCL and clove bud powder fibers

The antibacterial activity of the clove powder on the PCL nanofibers was evaluated. The antibacterial tests showed that after incubation for 24 h at 37 °C, the PCL nanofibers with 200 mg of clove bud powder had a minimal effect on lowering cell viability of *E. coli* (DH5 α) (see Figure 5.3). This could be due to the very low concentration of clove bud powder that was incorporated into the nanofibers. It should be mentioned here that the clove bud powder did not dissolve completely in the mixture of the solvents (DCM: DMF). This resulted probably in an even lower concentration of the clove bud powder, which could be attributed to the complex mixture of hydrophilic and hydrophobic chemical compounds contained in clove buds. The main component of the clove bud oil is eugenol (49.71%) (C10H12O2 2-Methoxy-4-(2-propenyl) phenol) and is the compound most responsible for the aroma and strong antimicrobial activity. The other important component in the clove bud oil is caryophyllene, limonene, eucalyptol, methyl salicylate, chavicol, copaene, alloaromadendrene, germacrene D, aguaiene and d-cadinene [19]. As it was previously determined that the MBC concentration of clove bud powder was 200 mg, it was necessary to use the TGA to determine the amount of clove bud powder in the PCL fibers which was 3.3865 mg (See Figure 5.4), which is less than the MBC. This finding corresponds to the antibacterial activity results and it is reasonable to assume this, because the

clove powder didn't dissolve in the mixture of (DCM: DMF) as solvent and the most of the clove powder stuck inside the syringe during the electrospinning process.

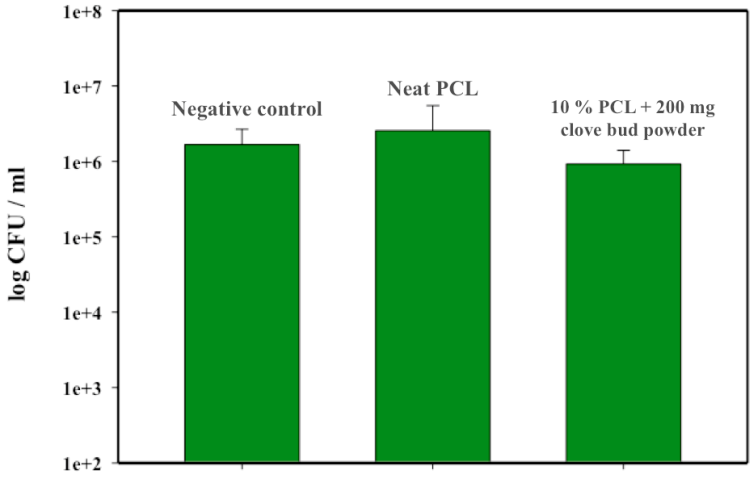

Figure 5.3 The antimicrobial activity of PCL + clove bud powder fiber

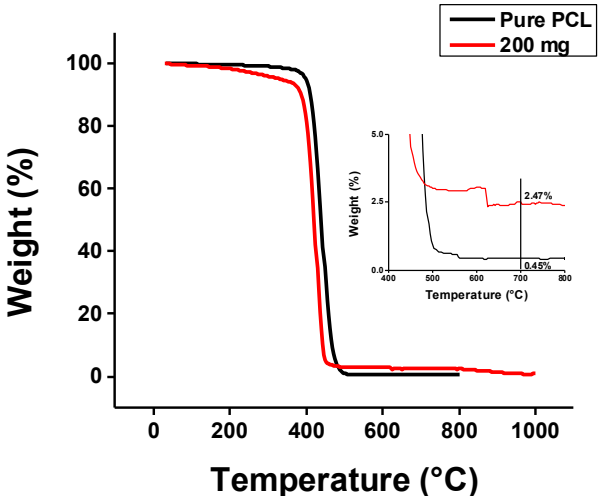

Figure 5.4 TGA thermal of PCL fibers with clove bud powder

Conclusions

The results of this study showed that clove bud powder exhibits strong antimicrobial activity against food borne pathogens such as *E. coli* (DH5 α) in vitro. The clove bud powder without sterilization showed a higher MBC value against the *E. coli* strain than that of the clove bud powder with sterilization. The antimicrobial activity of clove bud powder did not show a strong antimicrobial effect when incorporated into PCL electrospun fibers, which was attributed to the improper dissolution of powders in the solvents (DCM: DMF). However, our study confirmed that there is a notable antimicrobial potential for clove bud powder against *E. coli* (DH5 α). The practical availability of clove bud powder coupled with its production costs render its use as an antimicrobial agent possible as well as its incorporation into food packaging materials.

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CHAPTER 6

Development of Antibacterial Structures and Films Using Clove Bud Powder[2]

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Abstract

The demand for high quality food and the packaging that protects it has been the driving force of the food industry in the last decade. Antimicrobial packaging has been at the forefront in response to foodborne microbial outbreaks, providing a safe and effective method in protecting and delivering food to the consumer. However, some antibacterial materials used to package food have shown toxicity and adverse effects on human health. Interest has now turned towards developing natural materials in creative ways to protect food. In this study, clove bud natural plant powder (Syzygium aromaticum) was analyzed for determining its antimicrobial activity against Gram-negative *E. coli* (DH5α) and Gram-positive *L. innocua* (LSPQ3284) and *S. aureus* (54-73) microorganisms. The lowest concentration of clove bud powder preventing the growth of a microorganism after 24 h incubation was considered as the minimum inhibitory concentration (MIC) and the values of the minimum bactericidal concentration (MBC), concentration (MBC) was determined as well. The clove bud powder was prepared and coated onto low-density polyethylene (LDPE) films to evaluate its antimicrobial activity for food packaging applications. It was found that the clove bud powder inhibited the growth of the tested microorganisms. An LDPE film embedded with the clove bud powder by coating twice showed the best antimicrobial effects against *E. coli* bacteria.

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[2] **Submitted to Industrial Crops and Products Journal**

Introduction

In recent years, increasing incidences of foodborne diseases have caused considerable concern in food packaging, distribution and storage [1]. Despite advanced mechanisms in sanitation and inspection of food production, microorganisms have continued to present potential risks during food processing, packaging, shipping, storage and distribution. This has resulted in major challenges in food safety and quality [2-4]. Several research methods are proposed to find the best possible solutions in limiting the problems related to foodborne microorganisms and food spoilage. Antimicrobial (AM) packaging is a recent development technique that incorporates an antimicrobial agent into polymer films that are then used as the primary packaging material [5-7]. These films are designed to inhibit the activities of targeted microorganisms [5-7]. The great majority of these AM agents are chemically based, and the main concern of using chemical AM agents is their possible side effects like acetic acid and benzoic acid anhydride. Many studies have shown the impact on human health in the continuous consumption of food applied with such chemical antimicrobial (AM) agents [8-1].

Recent research has begun to develop and employ naturally derived AM agents as a substitute for chemically based AM agents. These antimicrobial (AM) packaging systems are primarily based on natural antimicrobial (AM) additives like activity of essential oils (EOs) or/and plant extracts [10-11]. Herbal plant species and spices are known to contain a wide range of organic compounds capable of exhibiting AM activity [8-12]. These compounds are secondary metabolites, which often have antimicrobial properties. The antibacterial properties of secondary metabolites were first evaluated in the late nineteenth century by De la Croix (1881), who employed essential oil vapors in his work [13]. The antimicrobial activity of plant-derived compounds against various pathogens, tested individually and in vitro, is well documented in literature. However, the results reported in numerous studies are difficult to compare directly as contradictory data have been obtained for the same antimicrobial compound [14-17]. Three principal factors can influence the outcome of the antimicrobial tests when used with EOs of plants and their compounds: the composition of the sample tested (type of plant, geographical location and time of the year), the microorganism (strain, conditions of growth, inoculum size, etc.) and the method used for growing and enumerating the surviving bacteria [18]. Interestingly,

some EOs such as thyme oil, clove bud oil, cinnamon leaf oil and other EOs and their compounds like thymol and eugenol have shown toxicity, particularly to vulnerable people like the very old, the very young, and pregnant women [19]. This toxicity in essential oils may not only be effective when consumed internally but also when applied externally (or inhaled) [19-20]. Toxic reactions can be felt immediately and range from dizziness, nausea, and allergic reactions to exhaustion, epilepsy and even death [19,20].

Past research has focused on the use of AM agents derived from naturally based plant extracts and their EOs but none have centered upon the AM activity of the ground plants themselves. To our knowledge, there hasn't been a study comparing the antimicrobial performance of the original ground plant powders and their essential oils in packaging films. Consequently, the purpose of this study is to examine the antimicrobial activity of clove bud powder against one Gram-negative bacteria *Escherichia coli (E. coli)* (DH5α) and two Gram-positive *Listeria innocua (L. innocua)* (LSPQ3284) and *Staphylococcus aureus (S. aureus)* (54-73), to determine its minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and its efficiency when coated onto or incorporated into packaging polymer films or sheets.

Clove is also known as Myrtaceae, which belongs to the myrtle family, and is an important aromatic spice. Clove is broadly cultivated in Madagascar, Sri Lanka, Indonesia and the south of China. It is extensively used in the pharmaceutical industry, especially in medicinal preparations for gums and teeth [21-22]. Several studies showed that clove oil possess high antimicrobial properties against important human pathogenic microorganisms and microorganisms that cause food spoilage due to a high concentration of its active compound, eugenol [22-26]. In a previous work, the antimicrobial activity of powdered plants from sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf were investigated. In some cases, the ground plants stopped the growth of the *E. coli* (DH5α) bacteria [27-28]. It was also noted that upon comparison, the clove bud powder possessed the best antimicrobial activity in killing all the bacteria at the MBC level of 200 mg/ml [28]. The next series of tests were conducted to ascertain the effect of the AM activity in the clove bud powder when blended to a polymer and to establish its potency. The antibacterial activity of the clove

powder when incorporated in PCL nanofibers was evaluated and antibacterial tests showed that after incubation for 24 h at 37 °C, the (10% (w/v)) PCL nanofibers with 200 mg of clove bud powder had a minimal effect on lowering cell viability of *E. coli* (DH5α) [28]. This could be due to the very low concentration of clove bud powder that was incorporated into the nanofibers because of the sedimentation in the electrospinning solution. It should be mentioned here that the clove bud powder did not dissolve completely in the solvents mixture of (DCM: DMF) used to dissolve PCL [28]. Therefore, it was necessary to study the antimicrobial activity of clove bud powder against different types of microbes and its effect when blended with polymers. Blending with other polymers or their coating may be a promising way to take advantage of the AM activity of clove bud powder and extend its application.

The objectives of this study were to evaluate the antimicrobial activity of clove bud powder and its ability to stop or inhibit the growth of foodborne pathogenic bacteria such as *E. coli, L. innocua* and *S. aureus*. To be specific, the MIC, which is the lowest concentration of clove bud powder that can prevent the growth of *E. coli, L. innocua* and *S. aureus,* and the MBC, which is the lowest concentration of clove bud powder required to kill a particular bacterium, after a 24 h incubation period, were evaluated. The size of clove bud particles was reduced and analyzed by using dry ring milling (Vibratory Disc Mill) and a Coulter LS200 Laser Granulometer in water, respectively. In addition, this study aimed to investigate the antimicrobial effects of coating of clove bud powder on LDPE films at two different levels or concentrations. Finally, the antimicrobial activity of the clove bud powder was investigated when blended with PCL via the solution-casting technique.

Materials and Methodology

Materials

The plant of clove bud was purchased from local stores in Montreal, Quebec, Canada. The low-density polyethylene LDPE (LD 100.BW) was supplied by ExxonMobil. The poly (ε -caprolactone) (PCL) CAPA 6800 was obtained from Perstorp Winning Formulas and the solvents

that were used; dichloromethane (DCM) and dimethylformamide (DMF) were obtained from the Sigma Chemical Company.

Preparation of clove bud powder and antibacterial testing

The clove buds were dried and ground using an electric mill grinder. The antibacterial tests on the clove bud powder were conducted in the Department of Microbiology, Infectiology, and Immunology at Université de Montréal. In each tube, a preparation of 150 mg of clove bud powder and 5 ml Phosphate-Buffered Saline (PBS) was mixed separately with *E. coli* (DH5*α), L. innocua* (LSPQ3284) and *S. aureus* (54-73), (approximately 10^6 CFU/ml), respectively. The experiment was carried out in triplicate. The tubes were then placed in the incubator at 37 °C for 24 h.

The time-kill curves of *E. coli* and *S. aureus* treated with clove bud powder were incubated at 37 °C for 4 h in PBS treated with 130 mg of clove bud powder. These were then sampled, diluted as necessary and viable counts performed at time 0, 10 min, 20 min, 30 min 1 h, 1.30 h, 2 h and then every 60 min up to 4 h on nutrient agar plates. The experiment was carried out in triplicate. Viable counts were read manually after 24 h incubation at 37 °C.

6.2.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC were determined by agar plate counting method (also called viable plate count or standard plate count), one of the most commonly used techniques for enumeration of bacteria and the lowest concentration showing at which there is no growth of organisms on the agar plate was regarded as the MBC. In the testing tubes, each bacterial culture (approx. 10^6 CFU/ml) *E. coli*, *L. innocua* and *S. aureus*, was inoculated separately into a rich medium of Brain-heart infusion (BHI) broth in the presence of different concentrations of clove bud powder and incubated at 37 °C with shaking for 24 h. Then six serial dilutions of PBS prepared for each tube (10^{-1} to 10^{-6}) for each sample. Then 100 µL from the final solution was added to a first eppendorf

containing 900 µL of PBS, where the dilution is 10^{-1}. Next 100 µL was taken from the first eppendorf and added to the second eppendorf also containing 900 µL of PBS and so on and so forth until 100 µL is added to the last eppendorf of 10^{-6} dilution. Three 10 µL samples were taken from each dilution and droplets are applied onto the agar plate. This was also performed for the control tubes, which were containing only bacterial culture (i.e. without clove bud powder). The MIC is defined as the lowest concentration that will inhibit the growth of microorganisms, after incubation for 24 h. To determine the MBC, l00 µL was taken from each initial bacterial sample (approx. 10^6 CFU/ml) after incubation for 24 h, and spread across the surface of an agar plate to dry before incubation for 24 h at 37 °C. A blank control plate was also prepared in exactly the same way except that no clove bud powder was used. All experiments were carried out in triplicate. The MBC is defined as the lowest concentration that completely kills 99.9% of bacteria for 24 h. [29-30-31].

6.2.4 Minimization clove bud particle size

The clove bud was ground in a dry ring mill, which is generally used for fine particle size reduction. A sample of 3 g of clove bud powder was ground for 10 min and 20 min in the Vibratory Disc Mill to reduce the particle size. The particle size distribution of clove bud powder was analyzed by scanning under an electron microscope (SEM) (FESEM, Hitachi S-4700), and Coulter LS200 Laser Granulometer in water before and after grinding.

6.2.5 The impregnation of LDPE with clove bud powder

The LD 100.BW film was produced using a Single Screw Extruder. The two LD 100.BW samples were coated with one and two layers of clove bud powder respectively by using a hot press at the temperature of 100 °C for 30 min. Most of the clove bud powder was inserted into the film. Then, the amount of clove bud powder on surface of LDPE film was determined by using Thermogravimetric Analysis (TGA) Q5100 (TA Instruments, New-Castle, DE) tested under nitrogen (N_2) atmosphere from 25 °C to 800 °C at rate of 10 °C/min.

6.2.6 Antimicrobial activity of LDPE and clove bud powder film

In this case, the static method was used. In this method, the samples are formed into polymer films or sheets where the AM agents may be coated or incorporated into the polymer films or sheets. Antibacterial tests were conducted on the films produced with impregnation of clove bud powder. Both active and blank samples are cut out into discs of diameter ranging from 4 to 5 cm. ISO 22196:2011 standards require that the surface of contact between the two polymer layers be between 400 and 1600 mm^2. Blank discs should be sterilized by washing with 70% ethanol before the test. The two discs (one blank and the other active) are sandwiched together after 200 μL of 10^6 CFU/ml broth is deposited between them. Both films are rubbed against each other, so the surface is contaminated. These are placed in a petri dish, and incubated at 37 °C for a period of 24 h. The next day, both discs are quenched in a test tube with 2ml of pre-prepared saline solution (PBS). Each sample is made in triplicate, along with the negative control containing two blank discs.

6.2.7 Preparation of the PCL and clove bud by the solution-cast method

The PCL films were prepared by casting PCL solution in mixture of (DCM: DMF) (50:50) v/v at a concentration of 8wt%. The PCL and the solvent mixture of (DCM: DMF) were continuously stirred for approximately 24 h at room temperature until the pellets were fully dissolved. Then, 200 mg of clove bud powder was added to the PCL polymer solution and stirred using a magnetic stirrer for two hours. The mixtures were then poured into individual glass dishes where the solvent was allowed to evaporate at ambient temperature and pressure for 72 h under the laboratory hoods (fume hood). The thicknesses of the films were approx. 0.395-0.425 mm.

Antibacterial tests were conducted on the blend film of PCL using the astatic method. The static method ensures a better contact between the bacteria and film treated by supplying constant agitation of the sample in a suspension during the test period.

Results and Discussion

Antimicrobial activity of clove bud powder

The antimicrobial activity of clove bud powder was tested against three different types of bacteria, the percentage of the surviving cells was measured by viable cell counts, and the results are shown in Fig. 6.1. When compared with the control samples, the results show a complete eradication of the bacterium *S. aureus*, and a high reduction of bacterial survival was observed in *E. coli* (reduction of about 1.5 log CFU/ml) whereas a slight inhibition of the *L. innocua* was observed. The results of our study indicated that, *S. aureus* and *L. innocua* bacteria are from the same category of Gram-positive but they possess different behavioral growth patterns, which have varying resistance to clove bud powder. *S. aureus* was found to be the most sensitive to clove bud powder than *L. innocua*. It is difficult to predict the susceptibility of different strains of bacteria to AM agents because the results reported in different studies using EOs vary according to the strains within the same species and the bacterium tested. For example, De Martino et al. reported that two different strains of *Bacillus cereus* behaved differently when exposed to the same EOs and their singular components [32-33-34]. To properly identify the mode of action of AM agents, it is necessary to study the AM activity when tested against multiple strains and different species of microorganisms.

Moreover, this study showed that the clove bud powder has higher antibacterial activity in inhibiting the growth of Gram-negative (*E. coli*) than Gram-positive (*L. innocua*). Many studies indicated that Gram-negative bacteria like *E. coli* were the most resistant to essential oils and their compounds, that is because the differing compositions of the outer membranes (OM) of Gram-negative bacteria and Gram-positive bacteria [35-36-37]. The major components of the OM of Gram-negative bacteria are lipopolysaccharides (LPS), lipids, and proteins [35-18]. The Gram-negative bacteria have a thinner peptidoglycan layer, so eugenol; which is one of the major constituents of clove oil and is a member of the phenylpropanoid class of chemical compounds; can interact with the cell envelope readily. As Gram-positive bacteria do not have an LPS layer, this decreases the affinity of the antimicrobial system of eugenol and the bacterial interface [35-

37]. As a result, our study has indicated that the difference in resistance of the Gram-positive and Gram-negative bacteria reflects the need for further comprehensive studies in observing multiple strains and multiple species of microorganisms.

Time-kill curves can follow microbial killing and growth as a function of both time and the number of viable bacteria (CFU/ml) [38,39]. It was used to determine the real-time that the clove bud powder needed to begin to kill the bacteria. From Fig. 6.2 the clove bud powder for *S. aureus* and *E. coli* began to kill the two bacteria at 10 min and at 20 min showed a complete annhilation of the bacterium *S. aureus*, while for *E. coli* at 4 h some bacterial survival was observed.

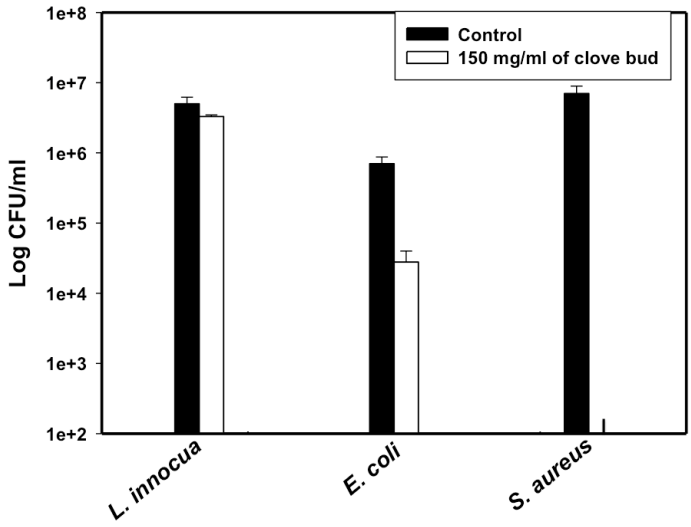

Figure 6.1 Antibacterial activity of clove powder

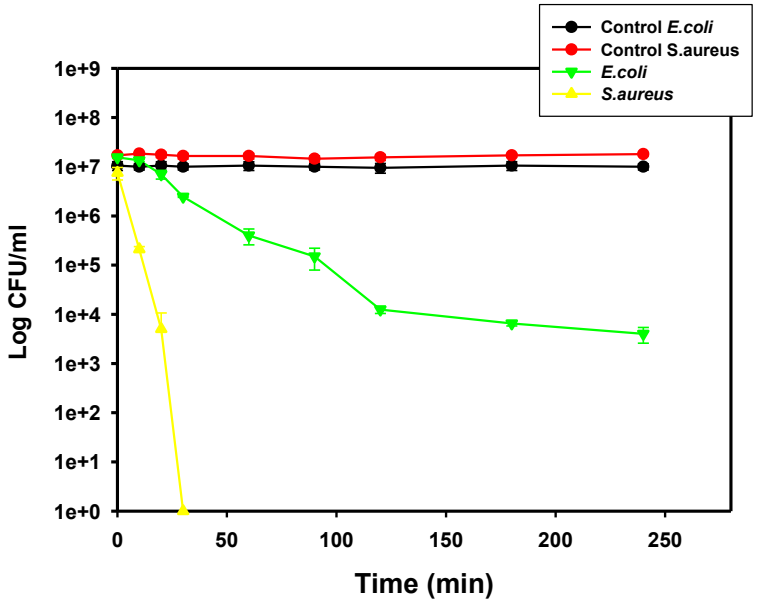

Figure 6.2 Time - killing curves

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and the MBC values of the clove bud powder demonstrate the growth of all test microorganisms, where the clove bud powder showed lower MICs and MBCs against *S. aureus* (Table 6.1) than *E. coli* and *L. innocua* by using agar plate methods. The MIC widely accepted and standard criteria for measuring the susceptibility of microorganisms to inhibitors. Many factors can affect the MIC values obtained, such as the conditions of growth, inoculum size and type of organism [18,38]. Furthermore, in our previous study, the MBC value for clove bud powder against the same bacterial strain *E. coli* (DH5α), inoculated into Luria-Bertani (LB) broth and agar, was 200 mg/ml [28]. In this study, the MBC values were obtained for clove bud powder that was inoculated into a Brain-heart infusion (BHI) broth and agar, was 230 mg/ml. The clove bud powder showed small differences in the MBC values, which was attributed to changing the culture medium from LB to BHI. This study showed that the clove bud powder has lower MIC and MBC values against *S. aureus* and *E. coli* than *L. innocua.*

Table 6.1 (MIC) and (MBC) of clove bud powder

Measured MIC and MBC	Model organisms		
	Gram-negative bacteria	Gram-positive bacteria	
	Escherichia coli	*Staphylococcus aureus*	*Listeria innocua*
MIC	190 mg/ml	100 mg/ml	510 mg/ml
MBC	230 mg/ml	120 mg/ml	550 mg/ml

6.3.3 Determination of particle size distribution (PSD)

The SEM provides information about the area projection, particle size and particle shape in a wide range of magnifications. Fig. 6.3 (A & B) shows the SEM micrographs of the clove bud powder obtained by (A – electric mill) and (B – dry milling) at 500 µm and 400 µm magnifications respectively. The images show that the clove bud particles were highly agglomerated and that they are present at different sizes and shapes. The particle size of the clove bud powder was measured by using Coulter LS200 Laser Granulometer before being ground and the average size was approximately 306 µm. After 10 min grinding, the average particle diameter of a particulate product is reduced to 86 µm but after 20 min grinding the average particle diameter of a particulate product increased again to 137 µm (see Figure 6.4). This later increase may be due to re-agglomeration, which means the particle size passes through a minimum and then increases with time as re-agglomeration begins to dominate.

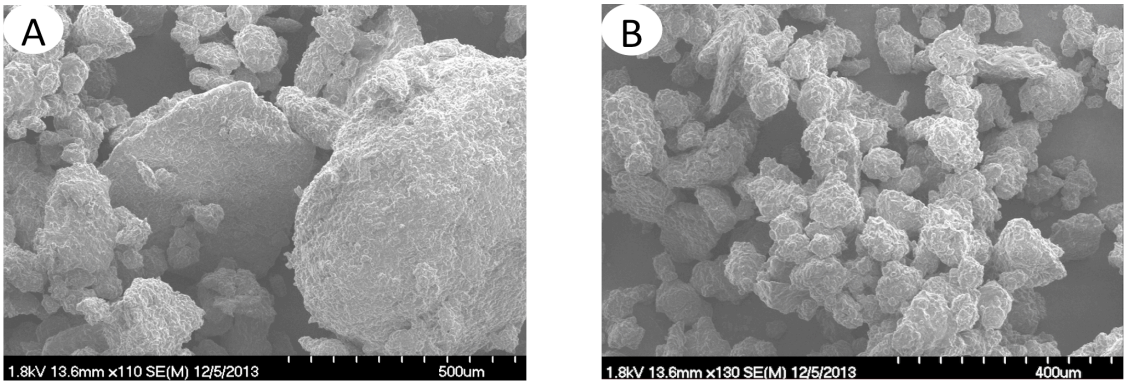

Figure 6.3 SEM micrograph of clove bud particles obtained from two different grinding procedures: A & B

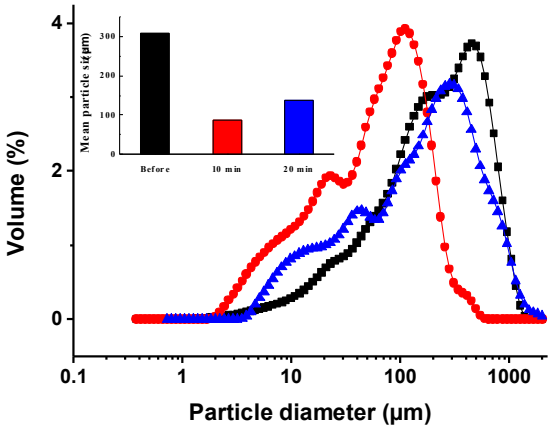

Figure 6.4 Particle size distribution of clove bud powder

6.3.4 Antimicrobial activity of LDPE and clove bud powder film

The antibacterial activity of the clove powder incorporated on the LDPE film surface was evaluated. The antibacterial tests showed that, after incubation for 24 h at 37 °C, the clove bud powder coated with two layers on the LDPE film surface (second concentration, double coating, Fig. 6.5) was the most effective antimicrobial agent against Gram-negative bacteria, *E. coli*, when compared to the LDPE film surface coated with one layer (first concentration) of clove bud powder. The LDPE film coated with one layer also showed good antimicrobial effects by inhibiting viable numbers of *E. coli* by approx. 2.5 log CFU/ml (see Figure 6.5). This could be due to the high concentration of clove bud powder that was impregnated into the LDPE film. It is imperative to determine the amount of clove bud powder on surface of LDPE film by using TGA. From Fig. 6.6, the amount of clove bud powder for one layer (first concentration) was determined to be about 118 mg on the surface of LDPE film. For the two layers (second concentration) of clove bud powder, it was 350 mg, which is more than the MBC of the clove bud powder 200 mg [28]. This finding corresponds to the results of the antibacterial tests. Our study confirmed that there is notable antimicrobial potential in clove bud powder against *E. coli*. The low production costs and the ready availability of the clove bud, render its use as an

antimicrobial agent as an eminently possible alternative to an EO by incorporating into food packaging materials.

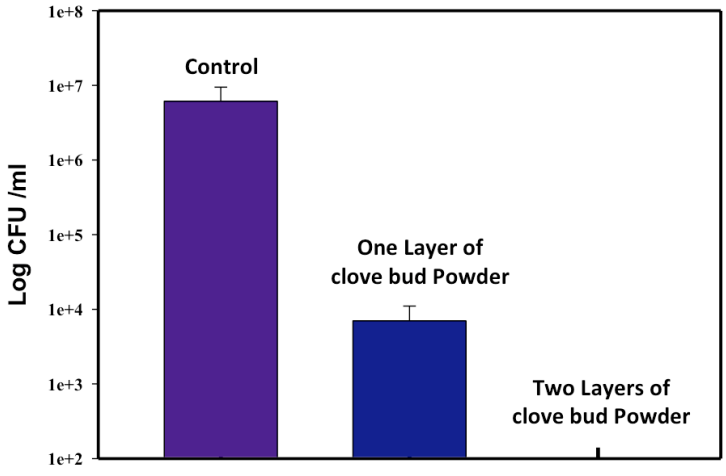

Figure 6.5 The antimicrobial activity of LDPE + clove bud powder film

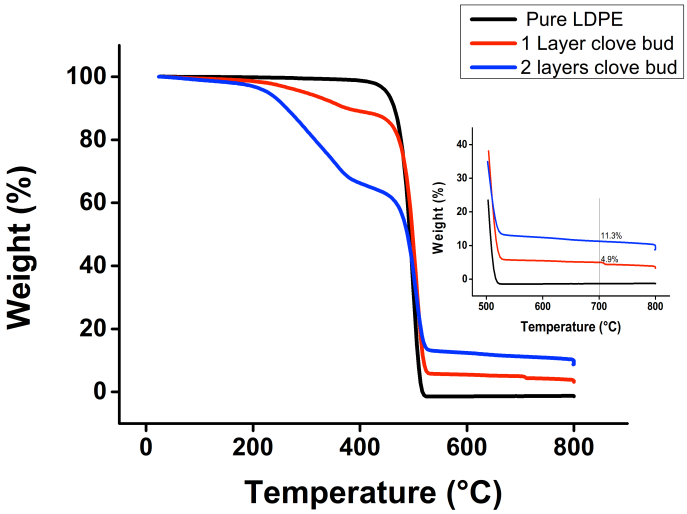

Figure 6.6 TGA thermal of LDPE film with clove bud powder

6.3.5 Antimicrobial activity of PCL and clove bud powder film

Various methods are employed in preparing antimicrobial films and coatings in food packaging solutions. The purpose of this study was also to evaluate the antimicrobial activity of clove bud powder blended with PCL in a film. Therefore, the investigation of the antibacterial effectiveness of the AM agents used focused against Gram-negative bacteria *(E. coli)*. The

results of the antibacterial activity, obtained using the static method, are presented in Figure 6.7. The results show that after, incubation for 24 h at 37 °C, the PCL/clove bud film has reduced the growth of *E. coli* by approximately 1.5 logs CFU/ml.

In general, antimicrobial agents such as EOs and their compounds themselves showed significantly higher inhibition against food-borne pathogens than when compared with EOs and their compounds, which were not incorporated into the polymer films or sheets [39-40]. Therefore, the main concept in using antimicrobial substances in polymeric matrices is to kill or inhibit the growth of microorganisms thus extending the shelf life and the safety of perishable packaged products [39-40]. However, the results can also be affected owing to many factors that can affect the efficiency of the antimicrobial agents when blended with polymers. These can include, the method of incorporation into the polymers, permeation and evaporation, controlled release, and the physio-mechanical properties of the packaging materials [41-42]. Until recently, researchers have investigated the activity of EOs and their compounds as antimicrobial agents, but there has not been any published work with regard to experimental herbal plants like the clove bud as an AM agent. The clove bud powder showed high antimicrobial efficiency against both Gram-positive and Gram-negative bacteria. Consequently, the aim of this study was to evaluate the antimicrobial activity of clove bud powder and its effect when coating and blending with polymers to gain insight about a possible application of the clove bud powder as an antimicrobial agent in food packaging. As a result, this study showed the potential use of clove bud powder for applications in antimicrobial packaging films or coatings. This study demonstrates that the clove bud powder as a natural AM agent and can be successfully incorporated into PCL film by using solution-casting technique and retain its inhibitory effect against *E. coli*. However, it would be interesting to see the clove bud powder blended with different polymers, using extrusion process, to determine if the clove bud powder will continue to retain its AM activity at high processing extrusion temperatures. This could also determine the adverse affects of the clove bud powder on the mechanical properties of the polymers after blending. Furthermore, before food samples are packaged using AM packaging material, the activity of clove bud powder films should be tested against variety of microorganisms.

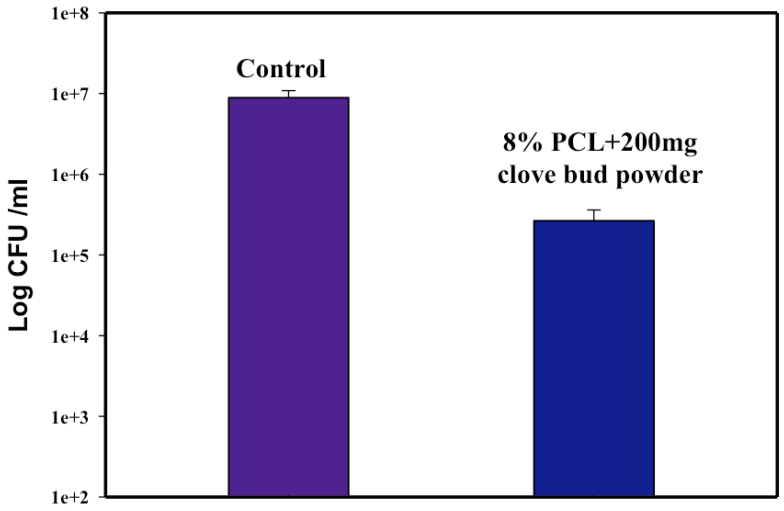

Figure 6.7 The antimicrobial activity of PCL + clove bud powder film

Conclusions

The findings of this study reveal that clove bud powder possesses considerable antimicrobial properties and can reduce the growth of *E. coli* and *S. aureus* strains; which represent common contaminants of food, environment and healthcare facilities. *S. aureus* and *E. coli* showed lower MIC and MBC values and were the most sensitive to the effects of the clove bud powder, in comparison with *L. innocua*. The degree of change in the values of the MBC was dependent on the culture media and strains tested. It was also found that the clove bub powder has ability to reduce the growth of the Gram-negative bacteria, *E. coli,* between 1.5 to 2.5 logs CFU/ml when it was coated onto the surface of LDPE films or blended with PCL via the solution-casting technique.

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CHAPTER 7 SUPPLEMENTARY RESULST

The release of thymol from films

The combination of AM agents into food packaging materials is aimed at extending the shelf life of packaged food products by preventing or inhibiting the growth of pathogens and food spoilage bacteria [149]. The release rate and migration of an AM agent is critical to maintain food quality and safety. For example, the rapid release of an AM agent from the packaging materials to the food surface may reduce the success of the AM agent as this might cause diffusion of the agent to internal parts of the food instead of the surface of the food, which is the place of the highest microbial growth and contamination [7]. On the other hand, if the release rate of the AM agent is slow, its inhibitory concentration may not be reached where food spoilage, and other factors affecting the food quality might occur affecting the safety of the food [7]. Therefore, controlled release of the AM agent from the packaging system to food surface is very important since it helps maintain the inhibitory concentrations of the agent at the critical food surface during storage periods.

The aim of this test was devoted to a characterization of the migration kinetics of thymol from LDPE film to the surface of the film over time by using FTIR–ATR spectroscopy test. The characteristics of the two grades of LDPE that have been used in this study are summarized in Section 3.1.1. The mixture of LD100BW resin and 2% of thymol were blended and produced by using a twin-screw extruder (see Section 3.2.1). After cooling, a white powdered material started to form on the film surface. It was assumed that the thymol had been incorporated into the resin and was in the process of migration to the film surface. The FTIR spectroscopy test was then performed in order to verify the presence of thymol in the film (see Section 3.3.4). Therefore, the FTIR spectroscopy test was applied to two different analytical samples over time, to measure the migration of thymol. The first method was conducted by measuring the migration at various times without cleaning out the thymol powder that was accumulated on the surface of the film, while in the second method, the samples were lightly dusted off by removing the thymol powder and measuring the migration time at various intervals.

The accumulation of the thymol powder on the surface of the film after cooling is attributed to thymol powder, which didn't blend very well with LDPE resin. This could be due to the polar structure of thymol that is incompatible with the matrix of polyethylene (non-polar). Following the cooling, instead of being trapped in the amorphous areas of the film, a large amount of thymol incorporated instantly migrated to the surface. The FTIR spectroscopy test was then performed every 2 h from the time of mixing, in order to verify if a portion of the powder had been incorporated within the matrix. The FTIR spectra of the samples were compared with FTIR spectra of pure thymol powder (see Figure 7.1). This comparison showed that, the FTIR spectra did not reveal any migration of thymol or display any changes in its concentration on the surface of the film. Moreover, the cross section analysis performed for the same film, confirmed a complete absence of the thymol powder in the interior of the matrix of the polyethylene. A sample of (LD100BW + 2% thymol) was set-aside in ambient air for two days. After two days passed, no powder was present on its surface, which indicated that the thymol evaporated easily in direct contact with the ambient atmosphere.

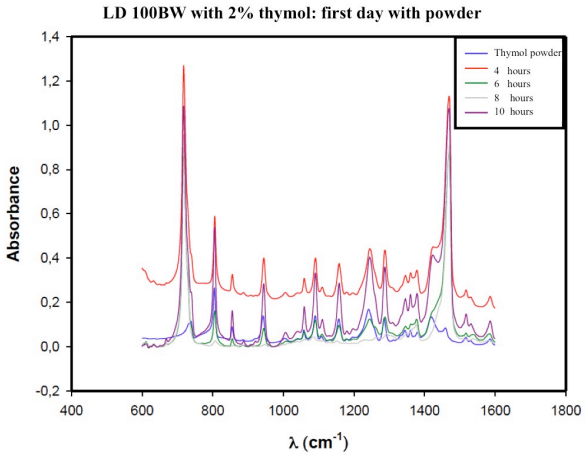

Figure 7.1 ATR-IR spectra of LDPE films with 2% thymol

To improve the quality of mixing of the thymol, other samples were prepared. The LD 3003 linear polyethylene resin and 2% of thymol were blended using the Brabender internal mixer at 190 °C and the mixing time was 8 min (see Section 3.2.2). When using the internal mixer for the preparation of the sample, an improvement in the quality of the mixture was noted due to the absence of crystals on the surface of thymol sheets. The sample of 2% of thymol was

analyzed also by FTIR–ATR spectroscopy to study the migration kinetics of thymol from the LLDPE to the surface of the film over time. Fig.7.2 shows that some small peaks were observed indicating the migration of thymol after 4 h and 6 h around 1700 cm^{-1} but disappeared after 12 h. As shown in Fig. 7.3 after a week the FTIR spectra test showed no migration of thymol to the surface of LLDPE sheet. It is reasonable to conclude, this was probably because the thymol migrated to the surface and then evaporated into the air.

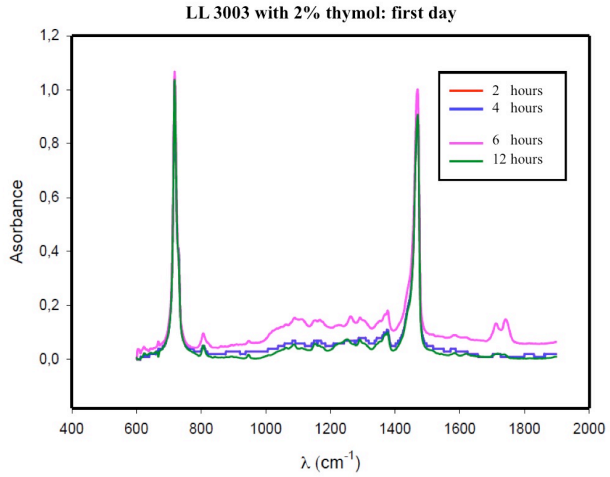

Figure 7.2 ATR-IR spectrum of LLDPE films with 2% thymol

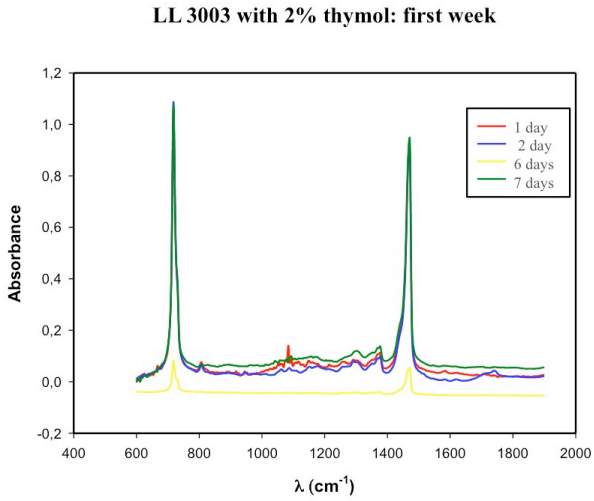

Figure 7.3 ATR-IR spectrum of LLDPE films with 2% thymol

Consequently, the results have indicated that even for this film, in which the mixing was better when comparing with the film produced by twin-screw extruder, there was no significant

migration of the powder to the surface of the sheet. This was most likely due to losing a significant quantity of thymol by evaporation during mixing in the Brabender. Therefore, the TGA tests were performed on pure thymol under nitrogen and air atmosphere to understand this phenomenon (see Figure 7.4). The results of which showed that the thymol rapidly evaporated, under nitrogen atmosphere than in air, because in the latter case the air was stagnant, whereas the nitrogen remained flowing through the TGA chamber. The actual evaporation of thymol occurred after exceeding its melting temperature (≈ 80 °C). To gain better insight on the rate of evaporation of thymol at constant temperature (condition of mixing in Brabander), two isothermal TGA analyses were performed at 65 °C (beginning of evaporation) and 190 °C (temperature of mixing). The results showed that the evaporation was very rapid and thymol at 190 °C completely evaporated and disappeared in an interval of 2 min (see Figure 7.5). These findings confirmed the absence of thymol in LLDPE films. The release rate of the AM agents from the packaging system is highly dependent on their volatility, which relates to the chemical interactions between the volatile AM agent and the packaging materials [12]. The higher release of thymol can be credited to its high volatility and weak interactions with the LDPE as a polymer matrix, during the integration of thymol into a polymer. On the other hand, the polarity and molecular weight of the AM agent has to be taken into consideration during integration of the AM agent into a polymer [150]. Antimicrobial agents with high molecular weights and low polarities are more compatible with LDPE polymers which is non polar [150]. Moreover, the molecular weight, ionic charge and solubility of different AM agents affect its rate of diffusion into the polymer [151]. Wong et al. (1996) compared the diffusion of the AM agents such as; ascorbic acid, potassium sorbate, and sodium ascorbate in calcium-alginate films at varying temperatures of 8, 15, and 23 °C [152]. Their study found that ascorbic acid had the highest diffusion rate and sodium ascorbate the lowest at all noted temperatures [152]. These findings were attributed to the different ionic states of the additives [152].

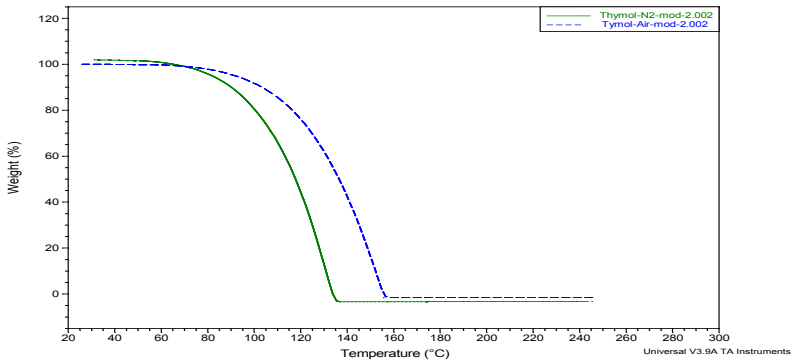

Figure 7.4 TGA curve of pure powdered thymol

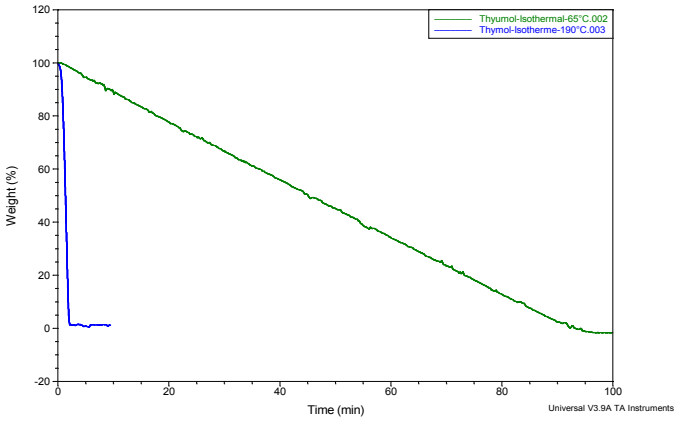

Figure 7.5 Isothermal TGA curves for pure powdered thymol

Furthermore, the antibacterial activity of LLDPE sheets contain 2% of thymol according to the same procedure described in the Section 3.4.2 of the static method and the sample of 2% of thymol showed slight reduction in the growth of *E. coli* (see Figure 7.6) owing to the very low amount of thymol that was incorporated into LLDPE sheets as explained above.

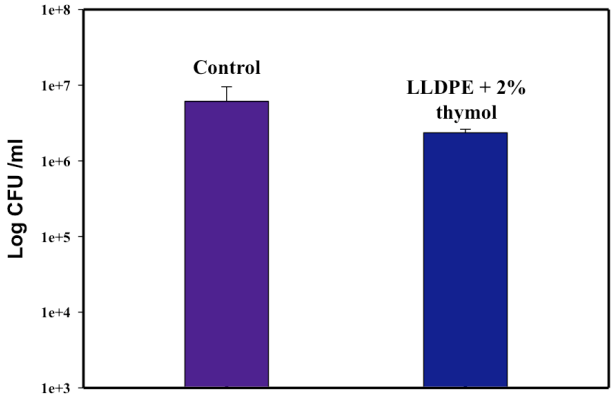

Figure 7.6 The antimicrobial activity of LLDPE + thymol film

In addition, the antibacterial activity of thymol was studied by using the process of electrospinning. The electrospinning technique is an efficient processing method for the manufacture of membranes with high specific surface areas, and can be exploited in several areas of application. This technique can be used with a variety of polymeric resins to produce fibrous membranes. There are many advantages in the use of the electrospinning process for natural AM agents like EOs and their compounds; as the nanofiber has a very large surface area, a considerable amount of the natural AM agent is not confined within the fibers, but is exposed outside of the fibers as well, thus exerting its useful higher AM effects [153]. Secondly, using the solution spinning method, the natural AM agent is uniformly distributed over and firmly bound to the surface of the nanofiber. Moreover, the functional properties of the natural plant extracts and/or essential oils are expressed in the nanofiber without loss even in high temperatures [153]. Nevertheless, fibers with EOs and their compounds have been studied only slightly [153]. The antibacterial activity of 15% PCL+200 mg of the thymol in the nanofibers mat, which has thickness around 0.132 – 0.732 mm and 0.208 - 0.330 mm thickness for the pure PCL mat was evaluated by following procedure of the dynamic method described in the Section 3.4.3. Antibacterial tests (see Figure 7.7) have shown, following incubation for 24 h at 37 °C, PCL nanofibers with 200 mg of thymol, had a minimal effect on the decrease of the cell viability of *E. coli*. That can be attributed to the evaporation of thymol during the electrospinning processing at room temperature. From these results, it can be concluded that thymol evaporated during processing and blending at high temperatures and applications at room temperatures, which can affect its efficiency as an AM agent. Otherwise, the antimicrobial properties of the

original plants like the clove bud powder can withstand relatively high temperatures and begins to degrade at around 250 °C which is markedly different of their EOs (see Figure 7.8). Fig. 7.8 shows the TGA test where the clove bud powder was dried at 110 °C for 15 min under N_2 gas, and the test completed from 100 to 1000 °C in air.

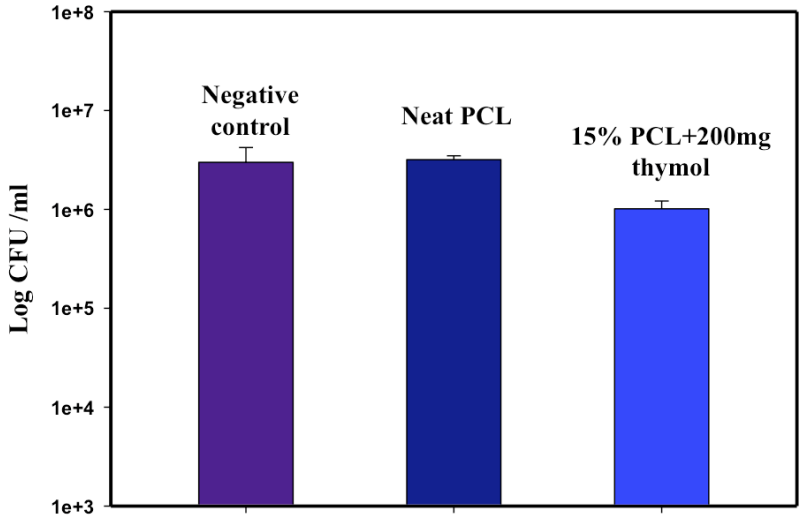

Figure 7.7 The antimicrobial activity of PCL + thymol fibers

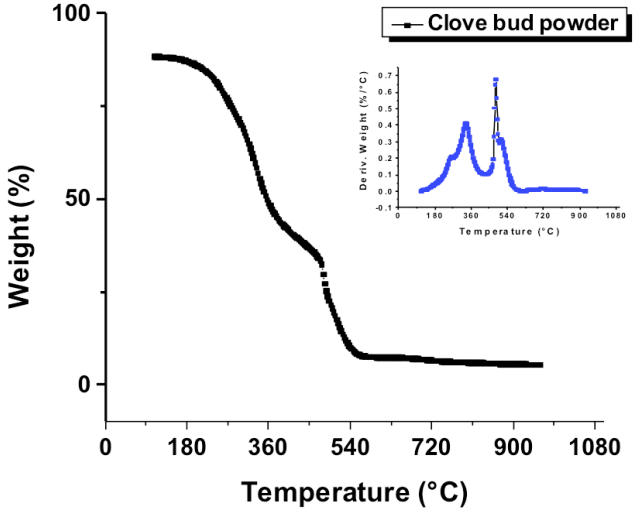

Figure 7.8 TGA thermal curve of the clove bud powder

CHAPTER 8 GENERAL DISCUSSION

Packaging materials provide a means to preserve, protect, merchandise, market and distribute foods. These materials are a key function in the way a product reaches the consumer safely without compromising quality. Active packaging now includes a broader concept, where the product, packaging material and the environment interact compatibly to extend the shelf life of food [154,155]. There are many different AM agents that can be incorporated into the packaging material to improve its functionality. Essential oils and plant extracts derived from herbs contain many compounds such as thymol, linalool, eugenol and carvacrol, exhibit a broad AM range against different pathogenic and spoilage microorganisms including Gram-negative [156,157] and Gram-positive species [158,159]; as well as against yeast [160] and molds [161]. While the antimicrobial properties of EOs and plant extracts have been known and used for many years, and consequently, previous studies have focused on the AM agents present in the EOs extracted from plants like thyme, clove, rosemary, cinnamon and oregano, but not on the ground plant themselves.

The migration of packaging components into food consumed, has received significant attention that has generated improvements in the antimicrobial properties of AM agents in recent years [43,44,45]. The antimicrobial properties of EOs like thymol have been reported in many studies [76,79,96]. However, studies on the packaging materials that can release active compounds are extremely limited. Therefore, the first part of this dissertation is aimed at understanding and exploring the release kinetics of thymol from (LDPE and LLDPE) films to the surface of the film over time by using the FTIR–ATR spectroscopy test. Our findings have indicated that after incorporating the thymol into LDPE polymer by twin-screw extruder, the most amount of thymol accumulated on the surface of the film due to the high polarity of thymol making it less compatible with the LDPE polymer, which is non polar. The FTIR spectroscopy test demonstrated that there was no migration of thymol nor a change in its concentration on the surface. Moreover, the cross-section analysis confirmed the complete absence of the thymol powder in the interior of the matrix of the polyethylene. Even though, improvement in the quality of mixing the thymol and LLDPE by using Brabender, there was no significant migration

of the powder to the surface of the film, however, we lost a significant quantity of thymol through evaporation. As a result, the higher release of thymol can be accredited to its high volatility, polarity and molecular weight, which led to weak interactions with the LDPE as a polymer matrix, during the integration of thymol into a polymer. In addition, the results of the antimicrobial tests of LLDPE + thymol films and PCL + thymol fibers illustrated that the entire amount of thymol used, was lost during processing, except for some residual sediment (<1%) which showed a slight reduction on the growth of *E. coli*. As EOs and their compounds are delicate and cannot withstand high temperatures, they need to be blended with polymers even for applications at room temperatures. Also, these EOs and their compounds have shown varying degrees of toxicity and adverse effects on human health. Therefore, it was decided to work with the plant powders themselves instead of the essentials oils.

The initial part of this study began with evaluating and comparing the antimicrobial activities of various ground, powdered plants such as sage, clove bud, clove leaf, lemongrass, black mustard seed, wild mint leaf, and thyme leaf, to select the ground powdered plant that showed the greatest antibacterial effects in the reducing or preventing the growth of *E. coli*. The results in this study presented that the clove bud powder has the greatest antibacterial activity, which would enable it eradicate all bacterial cells. That could be attributed to the two major constituents in clove oil, eugenol and beta-caryophyllene, where the high concentration of eugenol is responsible for the strong antimicrobial activity [66,67,69]. Moreover, the clove bud powder was tested against Gram-negative (*E. coli*) and Gram-positive (*S. aureus* and *L. innocua*) bacteria and the results illustrated that the clove bud powder has higher antibacterial activity in preventing and inhibiting the growth of Gram-positive (*S. aureus*) and in Gram-negative (*E. coli*) than Gram-positive (*L. innocua*). Furthermore, this study showed that the clove bud powder has lower MIC and MBC values against *S. aureus* and *E. coli* than *L. innocua.* The MIC which is the lowest concentration of clove bud powder that can inhibited the growth of *E. coli, L. innocua* and *S. aureus* on the agar plate. The MBC values were measured by using different test conditions and using two culture media (LB and BHI). The MBC values were obtained for the clove bud powder that was inoculated into LB broth and agar, without sterilization and with two different methods of sterilization (an autoclave at 121 °C for a period of 20 min and under UV Lamp with 254 nm wavelength for 20 min). The clove bud powder exhibited different MBC values against

E. coli. The MBC values of the clove bud powder without sterilization was 200 mg/ml and with two different methods of sterilization were ≥ 50 mg/ml. Our findings also revealed that the clove bud powder has different MBC values against *E. coli* by changing the culture medium from LB to BHI (200 mg/ml and 230 mg/ml) respectively. Our results substantiate what has been found in previous studies of EOs and their compounds, where there are many factors that can affect the antimicrobial activity and the MIC values, such as the microorganism (strain, conditions of growth, inoculum size, etc.), and the method used for growing and enumerating the surviving bacteria [162,163], the results of our study demonstrated that the *S. aureus* and *L. innocua* bacteria are from the same category of Gram-positive bacteria but they possess different behavioral growth patterns, which have varying resistance to the clove bud powder and the MIC and MBC values differed when changing the culture medium for the same bacteria strain (*E. coli*). Subsequently, the results of the time-kill curves of *E. coli* and *S. aureus* treated with of 130 mg of clove bud powder indicated that *S. aureus* is more highly sensitive to clove bud powder than *E. coli* and at 20 min all the *S. aureus* bacteria are killed.

During the last step of this project, 200 mg of clove bud powder was impregnated into PCL resin by using two different methods, the electrospinning technique and the solution-casting technique. This study revealed positive antimicrobial activity in inhibiting the growth of *E. coli*. Nevertheless, the PCL + clove bud powder electrospun fibers prepared did not show high reduction of the growth of *E. coli*, which was attributed to the improper dissolution of powder in the solvents (DCM: DMF) while, the PCL cast films showed a significant reduction of the growth of the *E. coli*. The LDPE film surface with a double coating of the clove bud powder exhibited high antimicrobial activity against the Gram-negative bacteria, *E. coli*, when compared to the LDPE film surface coated with one layer of clove bud powder. The LDPE film coated with one layer also demonstrated good antimicrobial effects by inhibiting viable numbers of *E. coli* (approx. 2.5 log CFU/ml). In general, experiments in testing the clove bud powder alone (without blending) do not necessarily provide a good indication of the potential value of the AM agent when incorporated into packaging materials. However, the results suggest that using clove bud powder with polymers could be effective, but the reductions in antimicrobial activity of clove bud powder when it is blended the polymers using different methods will produce varying results compared to its performance without blending due to the chemical nature of the films, the

process conditions like temperature, shearing forces, pressure and chemical interaction with the additives within film matrix (polarity, molecular weight, ionic charge and solubility). Experimental studies conducted with the clove bud powder in this dissertation showed that the clove bud powder is effective against the selected microorganisms (especially *S. aureus* and *E. coli* bacteria) even when blended with polymers.

CHAPTER 9 CONCLUSIONS AND RECOMMENDATION

Conclusion

In this dissertation, the FTIR spectroscopy tests results did not reveal any migration of thymol or a change in its concentration when studying the release of thymol from LDPE and LLDPE films to the surface of the films over time. The results of the TGA tests corresponds to the results of the FTIR spectroscopy tests, where the evaporation rate of thymol increased at high temperatures during blending with LDPE and LLDPE when using twin-screw extruder and Brabender. Except for some residual sediment (<1%), the entire amount of thymol used, was lost during processing.

The results of this study also showed that the clove bud powder exhibited strong antimicrobial activity against one Gram-negative bacteria *E. coli* and two Gram-positive *L. innocua* and *S. aureus*. The MIC and MBC values have clearly shown that the clove bud powder has higher antimicrobial activity against *S. aureus* than *E. coli* and *L. innocua.* This study revealed that the after grinding using the Vibratory Disc Mill, the size of the clove bud particle was reduced to 86 μm but then increased after prolonged grinding due to re-agglomeration.

Experimental studies conducted with different incorporation methods into PCL resin have shown that, the merger of clove bud powder into the PCL electrospun fibers prepared with 200 mg did not show high reduction of the growth of *E. coli* which was attributed to the improper dissolution of powders in the solvents (DCM: DMF). On the other hand, the PCL cast films prepared with 200 mg showed a significant reduction of the growth of *E. coli*, which means that the efficiency of the clove bud powder as antimicrobial agent is affected owing to changing the method of incorporation into the polymers.

The findings showed that, after incubation for 24 h at 37 °C, the clove bud powder with a double coating on the LDPE film surface was the most effective antimicrobial agent against Gram-

negative bacteria, *E. coli*, when compared to the LDPE film surface coated with one layer of clove bud powder. The LDPE film coated with one layer also showed good antimicrobial effects by inhibiting viable numbers of *E. coli* about 2.5 log CFU/ml.

Recommendations

The previous section has summarized what has been accomplished in this thesis concerning the development of antibacterial food packaging materials based on clove bud powder. For the continuation of this work and future research, as yet unexplored recommendations are listed below:

1) The findings from this study show that ground plants like clove bud powder can be highly efficient as a natural AM agent. However, further research is needed to observe the in vitro efficacy against pathogenic and spoilage microorganisms in food. Our results showed that there are many factors that can influence the outcome of the antimicrobial tests when clove bud powder is used as a natural AM agent such as the microorganism (strain, conditions of growth, inoculum size, etc.), and the method used for growing and enumerating the surviving bacteria. Therefore, it is also important to further investigate the antimicrobial activity of the clove bud powder at different growth conditions such as different temperatures and against different reference microorganisms.

2) This study also recommends a further reduction in the size of the clove bud particles, which can result in lower concentrations (MIC and MBC), of the clove bud that can be used to inhibit or kill the growth of microorganisms in food packaging materials.

3) In this dissertation, the main focus was on the developing antibacterial food packaging based on clove bud powder and the incorporation of clove bud powder as an AM agent into a polymer. This study revealed positive antimicrobial activity obtained in the results when the clove bud powder was blended into the polymers using different methods in varying results. It must be considered that certain factors can affect the antimicrobial effectiveness of the AM agents like the chemical nature of the films and the process conditions. Therefore, it would be interesting to see

the clove bud powder blended with different polymers, using the extrusion process, to determine if the clove bud powder will continue to retain its AM activity at high processing extrusion temperatures. This could also determine the adverse affects of the clove bud powder on the mechanical properties of the polymers after blending. Furthermore, before food samples are packaged using the AM packaging material, the activity of clove bud powder films should be tested against variety of microorganisms. Further investigation into the kinetics of microbial inactivation by AM films on real food at different growth conditions for example, different temperatures and against different reference microorganisms is equally important. The determination of the release kinetics of the clove bud powder is also recommended.

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